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*Some Physical Probes of Enzyme Structure in Solution**

SERGE N. TIMASHEFF

I. Introduction

The knowledge of the three-dimensional structures of a number of enzymes and other proteins, derived from X-ray crystallographic studies has greatly advanced our understanding in structural terms of the prop-

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erties of these macromolecules. Frequently, however, it becomes necessary to establish structural features of these molecules in solution and, in particular, to monitor changes in structure which may occur as a result of changes in environment or of interactions with various ligands such as substrates, inhibitors, or activators. Solution studies are obviously not capable of approaching the degree of resolution afforded by crystallographic techniques; they can, however, lead to the description of the general structural features of a biological macromolecule and pinpoint some particular details about the location and interactions of various specific groups on proteins. When we study enzymic reactions, we normally deal with systems in dilute aqueous solutions, in which the enzymes exist in a dynamic state as they undergo small conformational changes accompanied by changes in their interaction properties and in chemical reactivity. At present, the evidence seems to indicate that the structures of most proteins in the crystal state and in solution are not significantly different (*1*); furthermore, studies on enzyme–substrate and enzyme–inhibitor interactions in crystals have also made possible a test of various conformational changes which might accompany binding of these ligands (*2*, *3*). At present, the vast majority of enzymic systems are not amenable to direct crystallographic examination of conformation and the needed information must be obtained from solution studies. The X-ray crystallography of enzymes is treated by Eisenberg in Chapter 1, Volume I; therefore, it will not be discussed in the present chapter, except that it will be referred to when the crystallographic results are necessary for the interpretation of results obtained with solutions.

The problems which will be discussed in this chapter are concerned with the determination of enzyme secondary structure in solution, with the probing of side chain location and order, i.e., tertiary structure, and with perturbations of this order by the binding of ligands to specific sites on enzymes. The methods used in such studies are essentially of three categories: spectroscopic, such as ultraviolet and infrared absorption, optical activity, fluorescence, and nuclear magnetic resonance; thermodynamic, such as titration of specific ionizable groups, frequently with the use of their absorption properties in various spectral regions; and geometric, such as small angle X-ray scattering and various hydrodynamic techniques. No attempt will be made at an exhaustive presentation of the field but rather the salient features of various approaches will be presented, followed by a description of a few specific examples which

demonstrate what can be accomplished by any given technique in the present state of the art.

II. Secondary and Tertiary Structures

Recent years have witnessed an explosion of solution studies aimed at an understanding of the conformations of enzymes and other proteins. In these studies, the emphasis has been on spectroscopic techniques. In these techniques the fundamental observations are based on the interaction of electromagnetic radiation of a given frequency with some specific transitions of structural elements within the molecules. The nature of the investigated transition in each case is determined by the coincidence of the energy of the radiation with that of the transition. Thus, in the infrared spectral region, the radiation interacts with the modes of vibration of specific bonds; in the ultraviolet region, the interactions are with the electric and magnetic transitions of electrons in various groups on the protein. In complicated molecules, such as enzymes, the transitions are not independent of each other but usually they are coupled as a result of interactions between groups. Therefore, it is reasonable to expect that their energies, and thus band positions, will be affected by the conformations in which the specific groups are present within an enzyme. Secondary structure concerns the location in three-dimensional space and mutual interactions of the fundamental repeating units of the protein backbone, the peptide groups. This chapter will deal with the effects of conformation on the absorption of radiation by peptide groups in various spectral regions. Considerations of tertiary structure will involve similar effects on the spectra of side chain residues, more specifically, of differences in their environment, orientation with respect to other groups and interactions with the backbone chain and other structural elements of the protein molecule.

Structural analysis by spectroscopic techniques is complicated by a number of factors, the most prominent one being poor band resolution and the resulting band overlaps. The position of an absorption maximum does not represent necessarily the center of a band characteristic of a specific transition, and thus, conformation, but it is the result rather of the additivity of absorptions of closely located transitions which correspond to the various constituent conformations of a protein. The interpretation of any piece of spectroscopic data, therefore, must be undertaken with extreme caution; in fact, the most promising way of approaching solution structure is by the parallel application of several

unrelated techniques, with subsequent interpretation of the data in terms of a conformation that can fully account for all the observations made.

III. Infrared Spectroscopy

The infrared absorption spectra of polypeptides and fibrous proteins have been the object of extensive studies over a number of years. These were culminated by Miyazawa's theoretical analysis of the amassed data on the positions and shifts of the amide I and II bands in terms of a model based on a weakly coupled oscillator (*4–8*). As a result, it is possible now to relate small differences in band position to specific differences in the conformations of polypeptide chains. In protein spectra, the amide I band, located between 1600 and 1700 cm^{-1}, results mostly from C=O stretching, with small contributions from C—N stretching and N—H in-plane bending. The amide II band is located between 1500 and 1600 cm^{-1}; it corresponds to N—H bending and C—N stretching. In a polypeptide chain or protein, the exact positions of these bands are a function of chain conformation, namely, of the interactions between neighboring —C(=O)—N(H)— groups. These interactions may be of two kinds: (1) the vibrations of adjacent peptide groups may be coupled through the α-carbon atom; (2) they may be coupled across hydrogen bonds. In any given conformation, the transition moment to an excited state is a function of the coupling constants D_i multiplied by a phase factor (cos θ_i), where θ_i is the phase angle between the motions of the interacting oscillators. As a result, a spectral band with an unperturbed frequency ν_0 becomes split into several bands, whose frequencies ν are given by $\nu = \nu_0 + \Sigma_i D_i \cos \theta_i$. Since both D_i and θ_i are functions of the geometry and mutual organization in space of peptide bonds, the numbers and positions of bands observed for any transition in polypeptide chains are also a function of their conformations.

In synthetic polypeptides band assignment was greatly facilitated by dichroic measurements on oriented films; in globular proteins and en-

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TABLE I

AMIDE I BAND FREQUENCIES FOR PROTEINS IN VARIOUS CONFORMATIONS

Conformation	In H_2O (cm^{-1})	In D_2O (cm^{-1})	Films or crystals (cm^{-1})
α Helix	1652	1650	1652
Unordered	1656	1643	1658
APCP	1632(s)[a]	1632(s)	1632(s)
	1690(w)	1675(s)	1685(w)
Parallel β			1632 (calc)[b]
ν_0	1661	1654	1658

[a] s and w refer to strong and weak bands, respectively,

[b] From Krimm (7).

zymes in solution the advantages of orientation are not available and it becomes necessary to rely on band positions alone. The positions in solution of the amide I bands which correspond to various conformations have been established by studies of a number of model polypeptides and proteins in aqueous solution (*9*, *10*). These are listed in Table I. Infrared spectroscopy of proteins in solution is complicated by the fact that the usual solvent H_2O has a strong broad absorption band centered at 1650 cm^{-1}; as a result, routine solution measurements must be done in D_2O, which is essentially transparent in the spectral region of interest. This introduces uncertainties which stem from the facts that (1) deuteration of a protein may alter its conformation, and (2) it may result in band shifts in the infrared spectrum. While the first uncertainty must be eliminated by auxiliary experiments, the second one can be checked by comparing the solution spectra of proteins in D_2O with spectra of solid (crystalline) proteins suspended in some inert medium, such as Nujol, or cast as films out of aqueous solution. Examination of Table I leads to two important conclusions: (1) the band positions in three typical conformations, α-helical, antiparallel chain pleated sheet (APCP), or β, and unordered, are quite distinct; (2) the band positions in the α-helical and APCP conformations are essentially invariant whether the protein spectra are obtained on suspensions of crystals or on solutions of the proteins either in H_2O or D_2O. It is only in the unordered conformation that deuteration of the protein results in a band shift to lower frequency, probably reflecting interactions with solvent. Of particular importance is the presence of a second, weaker band at 1675–1690 cm^{-1} in the spectrum of the APCP structure; this band can be used to advantage diagnostically for the presence of that particular conformation in a protein. The parallel

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10. H. Susi, S. N. Timasheff, and L. Stevens, *JBC* **242**, 5460 (1967).

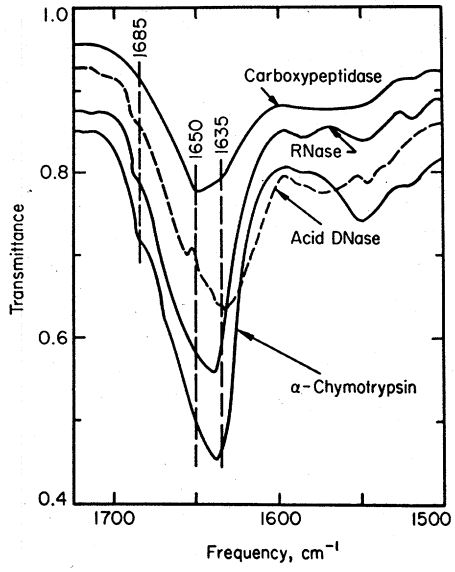

FIG. 1. Infrared spectra in the amide I band region of various enzymes in D_2O solution (arbitrarily displaced along the transmittance scale) (*11*, *12*).

β structure, while exhibiting a principal band in the same position as the antiparallel one, does not have the higher frequency absorption (*7*).

The conformations of a number of enzymes have been examined in the region of the amide I band absorption (*11–15*). Some typical spectra are shown on Fig. 1. Some of the significant features of these spectra merit comment. First, it is evident that the positions and shapes of the spectral bands differ for the various enzymes. Analysis of spectral details have permitted the assignment of some structural features. Both ribonuclease and α-chymotrypsin have absorption maxima at 1637–1640 cm^{-1} with marked shoulders at 1685 cm^{-1}. The shoulder at 1685 cm^{-1} is a definite indication of the presence of APCP conformation within these enzymes, in full agreement with the X-ray structural results (*16–18*); the shoulders

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around 1650 cm^{-1} are consistent with the presence of a limited amount of α helix, although they could not be used as diagnostic evidence for the presence of that conformation. Carboxypeptidase A, to the contrary, has a maximum at 1650 cm^{-1}, a pronounced shoulder at 1637 cm^{-1}, and no discernible band at 1685 cm^{-1}. This is consistent with the presence of both α-helical and β conformations, with a larger amount of the former. X-ray diffraction studies (*19*) have shown that this enzyme contains about 25–30% of α helix and about 18% of β conformation; the latter, however, is of both the parallel and antiparallel types, with a predominance of the former; from Table I, a band at 1685 cm^{-1} could have been expected, as a contribution of the antiparallel pleated sheet structure. This band, however, is quite weak and it is very probable that its intensity in carboxypeptidase A is too low to be resolved from the steeply falling edge of the strong lower frequency band. Another interesting example is afforded by the allosteric enzyme, acid deoxyribonuclease. This enzyme is known to be a dimer of two identical chains (*20*, *21*). Its secondary structure, however, is not known. Its circular dichroism and optical rotatory dispersion spectra (*22*) are characterized by weak overlapping bands, which suggest the presence of a mixture of conformations, with little α helix. Its infrared spectrum, shown in Fig. 1, is similar to that of ribonuclease; the 1685-cm^{-1} shoulder suggests that parts of the polypeptide chain in this enzyme are folded in an antiparallel pleated sheet structure. These examples indicate the extent of information on the secondary structure of enzymes which may be derived from infrared spectroscopy. It is evident that the information is very limited and only of a general nature. At best, this method can be used to corroborate or interpret results obtained by other techniques such as circular dichroism or optical rotatory dispersion as well as to ascertain whether significant conformational differences exist between a given enzyme in the crystal and solution states. Where the crystal structure is available, it is consistent with the general conclusions drawn from the analysis of the infrared spectra. The particular advantage of the infrared technique is that the spectra are not complicated by overlapping side chain absorption, a situation which gives rise to difficulties in the ultraviolet region.

While infrared spectroscopy cannot give more than a general indication

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of conformational features present within a protein, it can be used to advantage in studying changes in conformation induced by changes in medium or by interactions. A typical example is given by bovine carbonic anhydrase (*11*). The infrared spectrum of this enzyme at neutral pH displays an amide I band with a maximum at 1637 cm^{-1}. When the pH is lowered, the amide I band maximum shifts from 1637 to 1647 cm^{-1}. Such a shift is consistent with a transition from a structure containing a significant amount of β conformation to one richer in α helix, as suggested by optical rotatory dispersion and circular dichroism observations (*23*); the latter spectra, however, are so complex at neutral pH that they essentially defy conformational analysis. When the pH is raised to 12.0, the amide I band in the infrared spectrum of bovine carbonic anhydrase remains identical to that observed at pH 7.4, suggesting that no major changes in secondary structure occur, while a similar pH shift is accompanied by gross changes of the optical rotatory dispersion and circular dichroism spectra. These infrared results permit a better understanding of the observations that the availability of tyrosine residues to titration remains unaltered up to pH 12 (*24*), as well as of the reactivity of these residues with cyanuric fluoride (*25*), which is most probably related to small changes in the tertiary structure and the charge configuration of the enzyme. In the case of the human enzyme, a similar increase in pH is accompanied by significant changes in the optical rotatory dispersion and circular dichroism spectra (*23*, *26–28*), which could not be interpreted in terms of secondary structure because of the presence of overwhelming aromatic residue transition bands. In view of the infrared results, it is now possible to assign these changes in the ultraviolet region almost exclusively to changes in side chain transitions, that is, to changes in the tertiary structure.

The examples given above show the extent to which infrared absorption in the region of the amide I band may be useful in the general structural characterization of an enzyme and in the analysis of conformational changes. Other information of similar nature may be also obtained from other bands characteristic of peptide transitions. For example, the amide V band gives better resolution; unfortunately, it is very weak. This band is located at much lower frequencies and is related to out-of-plane NH

23. S. Beychok, J. McD. Armstrong, C. Lindblow, and J. T. Edsall, *JBC* **241**, 5150 (1966).
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bending (*6*, *29*). Fasman and Miyazawa (*30*) have found that for synthetic polypeptides the characteristic band positions are α helix, 610–620 cm^{-1}; APCP (β), 695–705 cm^{-1}; and unordered, 650 cm^{-1}. These bands are weak relative to amide I and II, and N-deuteration results in considerable shifts. While they have not been applied yet to structural studies of enzymes, their good resolution should render them useful for such studies, in particular as highly sensitive spectrophotometers become available.

IV. Ultraviolet Absorption of Peptide Groups

Amide groups undergo electronic transitions in the ultraviolet spectral region below 240 nm. The nature and origin of these transitions has been reviewed recently (*31*) and will not be discussed here. Since, in coupled systems such as a polypeptide chain, the interactions between the transition moments of neighboring groups are functions of intergroup distance and orientation, the exact positions and intensities of the bands are a function of conformation. As a result, it is possible to use the ultraviolet absorption spectra of proteins in the region between 190 and 230 nm to analyze their conformations. The theoretical analysis leading to band identification is similar (*31–35*) to that described above for infrared spectroscopy and will not be discussed here. The absorption spectra characteristic of the α-helix, APCP, and random conformations are shown in Fig. 2 (*36*). While the spectra of the unordered, or random, and β conformations are very similar, formation of the α helix is accompanied by strong hypochromism. In analyzing the ultraviolet spectrum of a protein in terms of conformation, it must be first corrected for the contribution of aromatic side chain absorption, since these groups also undergo transitions in the same spectral region. The resultant curve is

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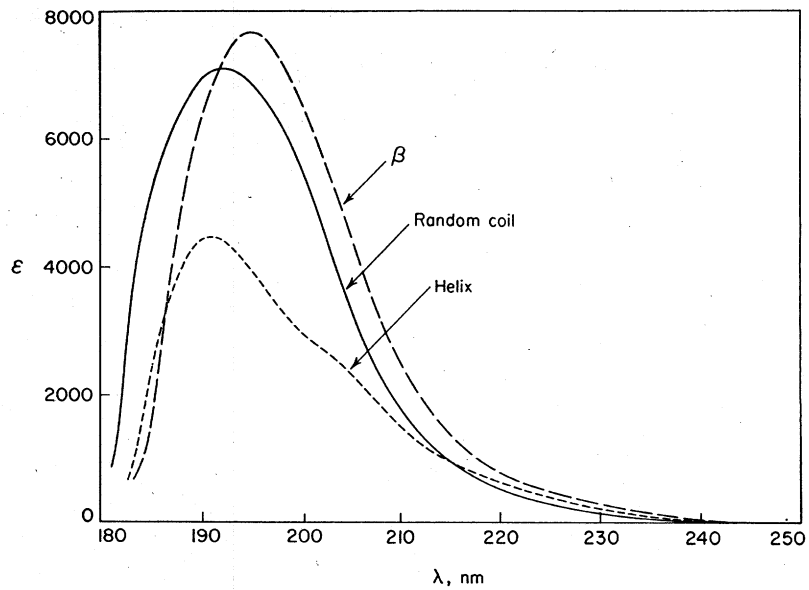

FIG. 2. Ultraviolet absorption spectra of poly-L-lysine in aqueous solution: random coil, pH 6.0, 25°C; α helix, pH 10.8, 25°C; β conformation, pH 10.8, 52°C [from Rosenheck and Doty (*36*)].

then fitted by a proper combination of the constituent conformational spectra. Such spectral analyses of protein conformation have been rather disappointing up to now, since it has not been possible to arrive at perfect fits in terms of the known spectra of polypeptides in the α-helical, APCP, and random conformations (*31*, *36*). This situation most probably reflects the fact that proteins contain a number of conformations other than the standard three. Certainly the regions devoid of long-range order within a protein structure cannot be identified with the random conformation of synthetic polypeptides in solution. These regions are highly organized and their structures are constrained by various interactions within the protein. The individual residues of randomly coiled polypeptides in solution, however, are in a state of Brownian motion, i.e., their mutual positions in space are constantly changing. Indeed, while the structures of individual molecules of a given protein are identical within the limits of thermal fluctuations in a randomly coiled synthetic polypeptide in solution, the probability is high that at any given instant no two molecules have identical conformations. The implications of these differences between randomly coiled synthetic polypeptides and the regions of protein structure devoid of long-range order will be discussed later in the examination of the pertinent circular dichroism spectra.

V. Circular Dichroism and Optical Rotatory Dispersion

While subject to some of the same limitations as ultraviolet absorption, as well as to greater theoretical uncertainty (*37*), the use of the optical rotatory properties of proteins has met with somewhat greater success. The optical rotatory properties of peptide bands stem from the same transitions as the ultraviolet absorption bands and convey essentially the same information. However, while absorption reflects electric moments only, optical activity results from a product of electric and magnetic moments (*38*). As a result, a transition characterized by weak absorption may have strong rotation, and vice versa. Thus, ideally, the two techniques should be complementary. Optical activity may be examined in two different ways: (1) by optical rotatory dispersion (ORD), i.e., the dependence of optical rotation on wavelength; and (2) by circular dichroism (CD), i.e., the difference between the absorption of left and right circularly polarized light. The advantages of CD are that it yields discrete spectral bands; furthermore, since the bands may be positive or negative, greater band resolution is afforded than in usual ultraviolet absorption which measures the sum of the absorptions of these two components of light. Optical rotation, being a dispersion phenomenon, gives rise to infinite bands and, thus, to strong overlaps. While this leads to difficulty in the resolution of bands in the region of transitions, this very overlap makes it possible to measure the optical rotation far from the wavelengths of particular transitions and may be used to advantage to follow changes in conformation without any specific knowledge of the structural features involved; this may be done by following either the variation in rotation at a single wavelength or the variation in the characteristic parameters of empirical relations, such as the Moffitt–Yang or Schechter–Blout equations (*39*, *40*), which describe the dependence of optical rotation on wavelength far from the region of transition. Circular dichroism and ORD are different manifestations of the same phenomenon and are related mathematically by the Kronig–Kramers transform (*41*, *42*). This interconversion is very useful in checking the internal consistency of measurements and as a help in band resolution (*12*, *43–46*).

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A. Secondary Structure

With the availability of highly sensitive instrumentation, which first permitted ORD (and later CD) measurements in the far ultraviolet, a vast amount of experience has been accumulated on the use of these techniques in the determination of protein and enzyme structure. We will confine our discussion strictly to the use of CD. The CD spectra of polypeptides in the α-helical and random conformations in solution were first reported by Holzwarth and Doty (*34*); the solution spectrum of the APCP structure was observed simultaneously on poly-L-lysine (*47*, *48*) and silk fibroin (*49*). The early work has been reviewed recently (*50*). Spectra typical of the three conformations in aqueous solution are shown in Fig. 3. Evidently these conformations are quite distinguishable in the case of a polypeptide in solution. The α-helix has two negative bands at 208 and 221 nm and a positive band at 191 nm; the APCP structure has a negative band at 217 nm and a positive band at 195 nm; the random conformation is characterized by a negative band at 196 nm, weak positive absorption at 218 nm, and very weak negative absorption at 235 nm. More recent studies have shown that the true situation in a protein may be much more complicated than a simple combination of these three spectra. Studies on polypeptide films have revealed that both the β and random (or unordered) conformations may give rise to more than one type of CD spectrum. Thus, Fasman and Potter (*51*) found in ORD studies that the APCP conformation may generate two general families of spectra which they called I-β and II-β. This finding was confirmed by CD experiments (*52*, *52a*). Typical spectra are shown in Fig. 3. They indicate that the nature of side chains and probably side chain interactions con-

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51. G. D. Fasman and J. Potter, *BBRC* **27**, 209 (1967).
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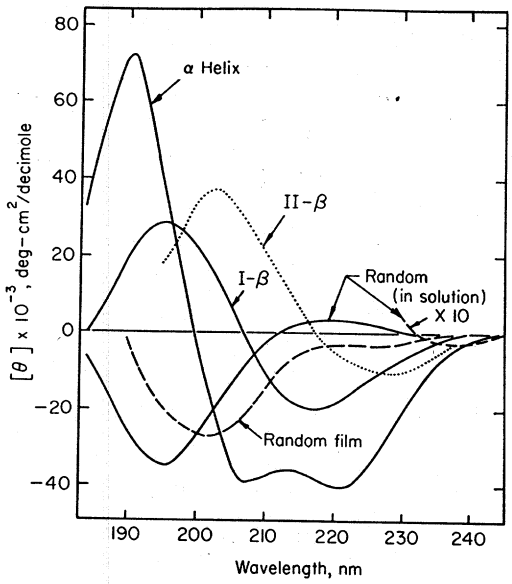

FIG. 3. Circular dichroism spectra of polypeptides in various conformations. α Helix: poly-L-lysine, pH 11.2, 25°C; I-β: poly-L-lysine, pH 11.2, after heating 20 min at 51°C; II-β: poly-*S*-carbobenzoxy-methyl-L-cysteine film cast from trifluoroacetic acid solution; random (in solution): poly-L-lysine, pH 7.5; random film: poly-L-lysine film cast from an aqueous pH 7.5 solution. [θ] is the ellipticity uncorrected for the refractive index of the solvent (*41, 47, 52*).

trol both band position and intensity. A similar situation exists in the case of the unordered conformation. Whereas, in solution, polypeptides in this conformation give spectra similar to that of poly-L-lysine, the spectrum changes drastically when an unordered polypeptide is cast as a film, as shown in Fig. 3; the strong negative band shifts to a higher wavelength, and the spectral features above 210 nm are replaced by a broad negative shoulder centered around 220 nm. This observation may be particularly pertinent to protein and enzyme structural analysis for two reasons:

(1) the unordered sections of globular proteins are not truly random chains, such as polypeptides in solution; in fact, they are organized in a definite geometry and subject to constraints imposed by the general folding of the molecule; in this respect, they are much better modeled by polypeptides in the state of hydrated films, in which the chain segments are also constrained and essentially immobile, than by random flight polymers.

(2) Denatured proteins give CD spectra much more reminiscent of

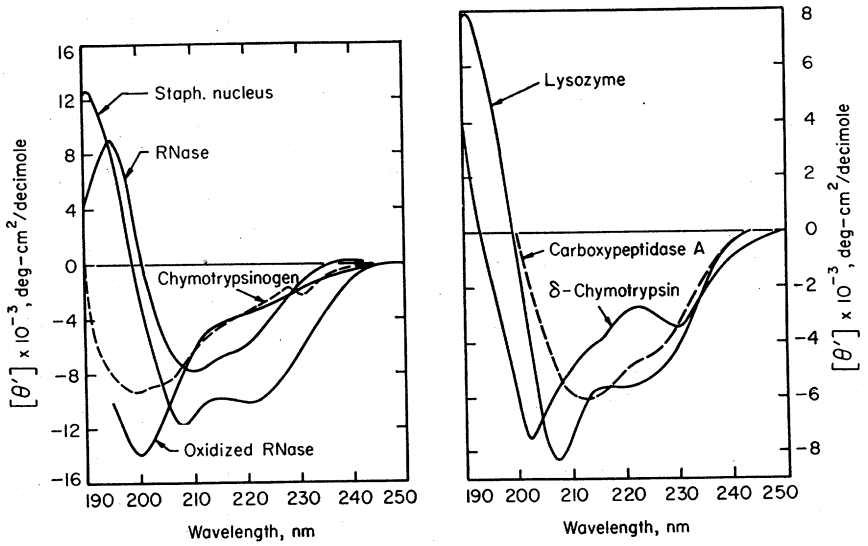

FIG. 4. Circular dichroism spectra in the far ultraviolet region of various enzymes. [θ'] is the ellipticity corrected for the refractive index of the solvent (*12, 13, 22, 52a, 71, 72*).

those of polypeptides in film form than in solution (*52a*); an example is given by the spectrum of oxidized ribonuclease shown in Fig. 4.

Further complications arise from the facts that band intensities and positions are sensitive to the nature of the environment (*49, 53*), to the extent over which the long-range order persists and to the state of association of polypeptides (*54*). It should also be pointed out that amide groups included in structures other than α-helical or β may give rise to CD spectra similar to those shown in Fig. 3 (*55–59*). The positions of CD bands associated with the various conformations are summarized in Table II.

Taking cognizance of the limitations of CD, it seems worthwhile to examine the extent to which enzyme structure may be probed by this technique by looking at some specific examples. In Fig. 4 are shown the

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TABLE II

CIRCULAR DICHROISM BAND POSITIONS FOR VARIOUS STRUCTURES

Structure	Band positions (nm) and sign
α Helix	221(−); 208(−); 191(+)
I-β	217(−); 195–198(+)
II-β	227(−); 198–203(+)
Random (solution)	235(−); 217(+); 196(−)
Unordered (film)	202(−); 220–230 sh(−)

ultraviolet CD spectra of ribonuclease, oxidized ribonuclease, lysozyme, carboxypeptidase A, δ-chymotrypsin, chymotrypsinogen, and staphylococcal nuclease. Just as in the case of the infrared region, these spectra are distinct from each other and are, in general, consistent with structures known from X-ray crystallography (*17–19, 60*). Greenfield and Fasman (*61, 62*) have made calculations of ORD and CD spectra of enzymes in terms of various compositions of α-helical, APCP, and random structures, using poly-L-lysine as a standard. For the proteins shown in Fig. 4, in the case of CD, they were able to account for the spectra between 205 and 240 nm in terms of the following compositions.

Ribonuclease: 9.3% α helix, 32.6% APCP, 58.1% random
Lysozyme: 28.5% α helix, 11.1% APCP, 60.4% random
Carboxypeptidase A: 13% α helix, 30.6% APCP, 56.4% random

In the first two cases the agreement is good with structures determined by X-ray crystallography (*17, 18, 60*). In the case of carboxypeptidase A, the CD analysis overestimates the contents of APCP structure almost by a factor of two, while underestimating the α-helical contents by an equal amount (*19*). This enzyme, however, is known to contain parallel β structure as well as antiparallel; this has not been taken into account specifically in the calculations. It is known, however, that the CD spectra of the two β structures should be almost identical (*35*). In the case of the staphylococcal nuclease, the spectrum shown in Fig. 4 is very typical for the presence of a significant amount of α helix (about 20%, when poly-L-lysine is used as a standard) (*22, 62a*). This value is in reasonable agreement with the results of X-ray diffraction studies (*63*). Discrep-

60. C. C. F. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Nature* **206**, 757 (1965).
61. N. Greenfield and G. D. Fasman, *Biochemistry* **8**, 4108 (1969).
62. N. Greenfield, B. Davidson, and G. D. Fasman, *Biochemistry* **6**, 1630 (1967).
62a. H. Taniuchi and C. B. Anfinsen, *JBC* **243**, 4778 (1968).
63. A. Arnone, C. J. Bier, F. A. Cotton, E. E. Hazen, Jr., D. C. Richardson, and J. S. Richardson, *Proc. Natl. Acad. Sci. U. S.* **64**, 420 (1969).

ancies which occur on occasion between estimates of the α-helical contents based on CD measurements and X-ray diffraction may be due to a variety of causes: First, the various factors discussed above may contribute to a wrong estimate. Second, in proteins, there may be individual residues with bond directions and angles identical to those found in ordered structures but which are not parts of such long-range structures. These will have transition moments and, thus, optical rotatory properties qualitatively similar to those of the given conformations, while in the structure deduced from X-ray diffraction they will lie in regions devoid of long-range order.

These examples indicate the degree of success that may be expected at present in the determination of the absolute secondary structure of enzymes by circular dichroism. While having serious limitations when used as a tool for the determination of the secondary structure of a protein, circular dichroism may be very useful in comparative studies of selected structures and of conformational changes which accompany enzyme activation or the binding of substrates. In such studies, it is frequently not essential to identify the exact nature of the conformational change which occurs; it may be sufficient to know the manner in which a specific band varies with a change in experimental conditions. This will be illustrated below by the specific cases of ribonuclease, carbonic anhydrase, and chymotrypsin, including its activation from zymogen.

B. Tertiary Structure

While CD spectra in the far ultraviolet reflect principally peptide bond transitions, and thus the secondary structure of a protein, important information on enzyme structure may be derived also from transitions of the side chains. These may reflect features of both the secondary and tertiary structures. Circular dichroism bands characteristic of amino acid side chains span the spectral region below 330 nm. Above 240–250 nm, there is no overlap with peptide bond transitions so that this region may be used to follow changes in side chain conformation and environment. Below 250 nm, the side chain transitions are superimposed on usually stronger peptide bands and therefore are not easy to distinguish, although, in some particular cases, they may become prominent features of the spectrum. The principal contributions are made by tryptophan and tyrosine residues, as well as by cystine disulfide bridges, although phenylalanine and histidine may also make significant contributions. The side chain CD spectra are usually complex as a result of the overlap of closely positioned bands. Furthermore, band positions, signs, and intensities may

vary for any given type of residue as a function of its environment. Factors which determine these characteristics of given bands include the nature of the ordered or unordered polypeptide fold to which the side chain is attached, the nature of its environment (polarity and asymmetry), the exact conformation of the side chain, the proximity of charged groups, the proximity of other groups undergoing electronic transitions with which the group in question may interact, and ionization of the residue in the case of tyrosine and histidine.

The positions of the CD bands of various amino acids were examined first by Legrand and Viennet (*64*), they have been summarized recently by Beychok (*45*, *65*). Incorporation of the amino acids into polypeptides results in band shifts which vary strongly with conformation. A typical example of the type of band shift which may occur is found in the results of Beychok and Fasman on poly-L-tyrosine (*66*) shown in Fig. 5. It is evident that a shift from the α-helical to the random conformation is accompanied by major changes in the spectrum: Not only are band inten-

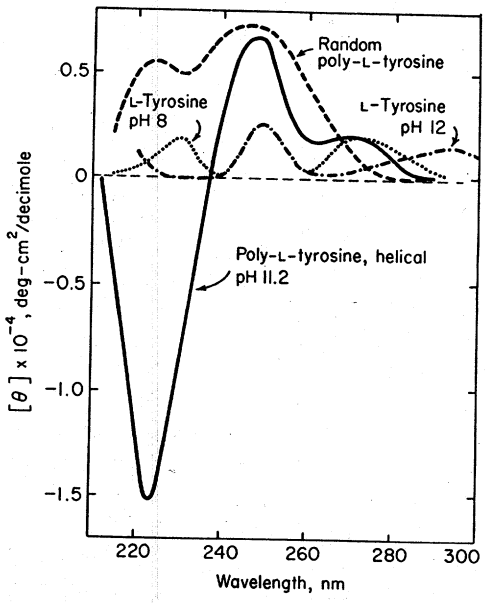


FIG. 5. Circular dichroism of helical poly-L-tyrosine, random poly-L-tyrosine (both at pH 11.2), and L-tyrosine at the pH values indicated [from Beychok and Fasman (*66*)].

64. M. Legrand and R. Viennet, *Bull. Soc. Chim. France* No. 3, 679 (1965).
65. S. Beychok, *Science* **154**, 1288 (1966).
66. S. Beychok and G. D. Fasman, *Biochemistry* **3**, 1675 (1964).

sities and positions altered, but even the sign of the absorption may change, as, for example, at 222 nm. Phenylalanine and its derivatives were found by Horwitz *et al.* (*67*) to give complex spectra between 250 and 268 nm; the band positions were essentially independent of structure and solvent, but their signs varied, being reversed both as a function of aggregation and residue conformation. A similar situation has been found to be true in the case of tryptophan. Peggion *et al.* (*68*) have determined the CD spectra of polytryptophan and of copolymers of tryptophan with γ-ethyl-L-glutamate, dissolved in ethylene glycol monomethyl ether. The spectra are shown in Fig. 6. In the near ultraviolet, positive bands at 293, 285, 278, and 266 nm reflect aromatic transitions. The progression of the curves below 240 nm from one dominated by a positive band at 230 nm and a negative one at 220 nm, characteristic of poly-L-tryptophan, to a spectrum characteristic of an α-helical structure, shows the overwhelming contribution which may be made by aromatic transitions in the far ultraviolet region as well. An example of such complex enzyme spectra is found in carbonic anhydrase (*23*, *46*). It is known that

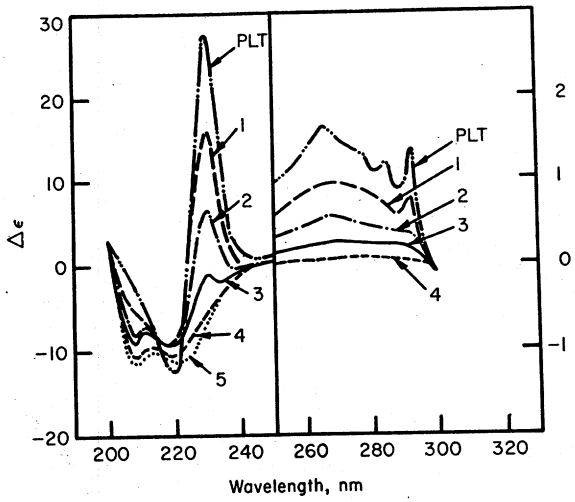


FIG. 6. Circular dichroism spectra of poly-L-tryptophan (PLT) and of copolymers of L-tryptophan and γ-ethyl-L-glutamate in ethylene glycol monomethyl ether solutions. The numbers on the curves refer to the following mole fractions of L-tryptophan in the copolymers: 1, 0.855; 2, 0.679; 3, 0.502; 4, 0.315; and 5, 0.159. $\Delta\epsilon$ is the difference between the absorptions of left and right circularly polarized light [from Peggion *et al.* (*68*)].

67. J. Horwitz, E. H. Strickland, and C. Billups, *JACS* **91**, 184 (1969).
68. E. Peggion, A. Cosani, A. S. Verdini, A. Del Pra, and M. Mammi, *Biopolymers* **6**, 1477 (1968).

the tryptophan copolypeptides are in the α-helical conformation (*68*, *69*). The spectrum of poly-L-tryptophan cast as a film has the same bands above 250 nm (*52*). The 230-nm band is negative, however, or of opposite sign to that found in solution (*68*); furthermore, the detailed structure of the spectrum is a function of film orientation. This indicates the strong effects which may result from the imposition of constraints on the random motion of polypeptide chains; such constraints are almost absent in solution but are prevalent both in films and in the interior of protein molecules.

Quite recently, Strickland *et al.* (*70*) have analyzed in detail the CD spectra of tryptophan derivatives in solvents of various polarities at room temperature and at 77°K. They were able to identify a number of overlapping dichroic bands which were assigned to various transitions. The low temperature CD spectra were almost twenty times as intense as those obtained at room temperature; the absorption weakness at 298°K reflects conformational mobility with cancellation of bands that correspond to different conformations. In the light of this observation, Strickland *et al.* analyzed the near ultraviolet CD spectra of chymotrypsinogen obtained at 298° and 77°K. The room temperature spectrum is shown in Fig. 7 (*71*, *72*). The bands above 285 nm have been assigned to tryptophan transitions on the basis of comparison with model compounds (*70*, *71*), as well as the observation that acetylation of three of the four tyrosines of this protein produced no changes in the CD spectrum as shown in Fig. 7 (*71*). Cooling of the chymotrypsinogen solution to 77°K resulted in only a slight change in the spectral intensity (*70*). The last observation, together with the fact that the optically active bands of tryptophan within chymotrypsinogen are red shifted with respect to the model compounds, led Strickland *et al.* (*70*) to the conclusion that in this zymogen the tryptophan residues are buried within the protein and thus have a relatively rigid position in space even at room temperature.

In the case of cystine residues, the situation is still more complicated. Not only is the CD spectrum a function of the various structural and environmental factors listed above, but also it is strongly affected by the dihedral angle about the disulfide bridge. Beychok (*45*) and Coleman and Blout (*73*, *74*) have investigated a number of disulfide-containing

69. G. D. Fasman, M. Landsberg, and M. Buchwald, *Can. J. Chem.* **43**, 1588 (1965).
70. E. H. Strickland, J. Horwitz, and C. Billups, *Biochemistry* **8**, 3205 (1969).
71. M. J. Gorbunoff, *Biochemistry* **8**, 2591 (1969).
72. M. J. Gorbunoff, in preparation.
73. D. L. Coleman and E. R. Blout, *in* "Conformation of Biopolymers" (G. N. Ramachandran, ed.), Vol. 1, p. 123. Academic Press, New York, 1967.
74. D. L. Coleman and E. R. Blout, *JACS* **90**, 2405 (1968).

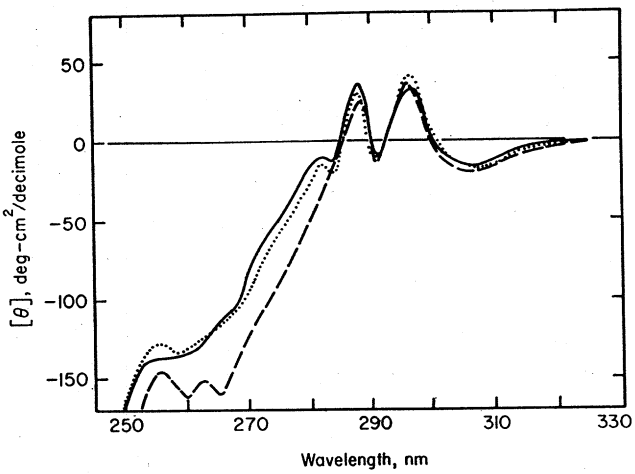


FIG. 7. Circular dichroism spectra in the near ultraviolet region of native chymotrypsinogen (*71*) in 0.001 *M* HCl(——), δ-chymotrypsin in 0.001 *M* HCl (*72*) (– –), and chymotrypsinogen with 3.2 tyrosines O-acetylated in pH 7.5 borate buffer (*71*) (.....).

compounds with the general conclusion that the principal transitions result in bands at around 200 nm and 260 nm, the signs being a function of the exact disulfide configuration. Beychok (*45*) has shown, furthermore, that with the proper configuration a disulfide bridge may give CD extrema at wavelengths as high as 350 nm. The contribution from disulfide rotation may be quite large. Coleman and Blout (*74*) estimated that for a polypeptide containing 25–40% α helix, 10% of the optical rotation near 210 nm may result from disulfide transitions if the chain contains two S—S bonds per hundred residues, a situation not unlike that found in many enzymes. They have also concluded that extensive cancellation of contributions from the 200-nm transition may occur in proteins because of variations of the dihedral angles of the S—S bonds.

From the preceding discussion, it is quite evident that side chain transitions give rise to a number of overlapping bands, which vary greatly in sign, position, and intensity, rendering the analysis of a protein spectrum extremely difficult. The positions of bands associated at present with various amino acid side chains are summarized in Table III. As a result, up to now, there has been only a limited success in the determination of tertiary structure from the side chain CD bands. Conversely, it is hoped that, as more data become available from properly controlled experiments on well-characterized model systems, the very complexity of the spectral patterns should become a useful tool in the determination of structural

TABLE III

IDENTIFIED CD BANDS OF CHROMOPHORIC RESIDUES

Residue	Band position (nm)	Residue	Band position (nm)
Trytophan	298–301	Tyrosine	275
	294–297	(unionized)	226
	290–293		
	287–290	(ionized)	292
	283–286		235
	277–278		
	265–270		
	222–225		
Cystine	260	Phenylalanine	268
	200		266.5
	(position varies with S—S dihedral angle)		264–265
			262
			259–260

details and, in particular, of changes in the tertiary structures of enzymes.

C. TYPICAL CASES

1. *Chymotrypsin*

One of the first indications that a structural reorganization takes place when chymotrypsinogen is converted to the enzyme was the observation of Neurath *et al.* (*75*) that this reaction was accompanied by a change in $[\alpha]_D$ from −79.8 to −60.3. That this did not reflect a major change in α-helical contents is evident from the observations of Raval and Schellmann (*76*) and Fasman *et al.* (*77*) that the Moffitt–Yang b_0 parameter did not change within experimental error during activation at pH 6.8–7.0. Extending the optical rotation dispersion studies into the far ultraviolet region, Raval and Schellman (*76*), Biltonen *et al.* (*78*), and Fasman *et al.* (*77*) observed that activation of the zymogen to the enzyme was accompanied by spectral changes in the region between 220 and 235 nm, where an apparently negative Cotton effect, centered at about 230 nm, became much more pronounced in the enzyme than in the precursor. The relation of this spectral change to molecular events

75. H. Neurath, J. A. Rupley, and W. F. Dreyer, *ABB* **65**, 243 (1956).
76. D. N. Raval and J. A. Schellman, *BBA* **107**, 463 (1965).
77. G. D. Fasman, R. J. Foster, and S. Beychok, *JMB* **19**, 240 (1966).
78. R. Biltonen, R. Lumry, V. Madison, and H. Parker, *Proc. Natl. Acad. Sci. U. S.* **54**, 1018 and 1412 (1965).

was clarified later in the elegant studies of Hess and co-workers (*79, 80*), who examined with a variety of techniques the problem of the detailed structural changes which accompany the activation of chymotrypsin. This section concerns solely ORD and CD analysis.

The far ultraviolet CD spectra of δ-chymotrypsin and chymotrypsinogen at pH 7.5 are shown on Fig. 4 (*60, 71, 72*). Both are dominated by broad negative absorption between 190 and 240 nm with a maximal amplitude between 197 and 207 nm. Between 220 and 235 nm both spectra contain a negative maximum near 230 nm and a negative minimum (located at 225 nm in the zymogen and 220 nm in the enzyme). The ellipticities of the extrema, however, are not identical. For the active enzyme, the ellipticity is more negative than for the zymogen, and the difference between $[\theta']_{230}$ and $[\theta']_{220-225}$ is more pronounced. At lower wavelengths, the amplitude of the CD is more negative in the precursor than in δ-chymotrypsin, suggesting that the enzyme structure contains somewhat more order than is found in chymotrypsinogen. The small differences in CD are also present in the spectral region of side chain absorption. Between 250 and 285 nm, the CD of δ-chymotrypsin is more negative than that of the precursor. Above 285 nm the CD spectra are essentially identical, indicating that activation has little effect on the environment of the tryptophan residues. Thus, a subtle conformational difference is revealed between chymotrypsinogen and the active enzyme. Hess and coworkers (*79, 80*) related these spectral changes to alterations in the conformation of specific amino acid residues by the following analysis.

The chemical difference between chymotrypsinogen and δ-chymotrypsin is known to be the absence of a dipeptide between residues 13 and 16 in the enzyme. Further peptide bond cleavage leads to the formation of α-chymotrypsin. Since the CD spectra of α- and δ-chymotrypsin are essentially identical in the 220–240 nm spectral region, the entire conformational difference between chymotrypsinogen and the enzymes must be related to the removal of the Ser 14–Arg 15 dipeptide. Crystallographic studies have shown that in α-chymotrypsin, at neutral pH, the α-amino group of Ile residue 16 and the carboxyl group of Asp 194 form an ion pair and then point toward the nonpolar inside of the protein molecule (*81*). Thus, from a comparison of chymotrypsin and chymotrypsinogen X-ray data (*82*), it appears that activation of the enzyme is accom-

79. J. McCann, G. D. Fasman, and G. P. Hess, *JMB* **39**, 551 (1969).
80. G. P. Hess, *Brookhaven Symp. Biol.* **21**, 155 (1969).
81. B. W. Matthews, P. B. Sigler, R. Henderson, and D. M. Blow, *Nature* **214**, 5089 (1967).
82. J. Kraut, H. T. Wright, M. Kellerman, and S. T. Freer, *Proc. Natl. Acad. Sci. U. S.* **58**, 304 (1967).

panied by a displacement of Ile 16 by a distance of 10 to 15 Å (*79*). In the absence of the positive charge on Ile 16, Asp 194 must point into the solvent (*83*), since the presence of a charged group within the low polarity interior of a protein molecule would result in an unfavorable free energy contribution of sufficient magnitude to destabilize the native structure of the enzyme (*84*). Thus, movement of Ile 16 on activation implies movement of Asp 194. Movement of Asp 194, in its turn, requires movement of Ser 195 (*80*), which is required for the catalytic activity of the enzyme. In the active enzyme it appears to be hydrogen bonded to His 57 which is also required for activity.

That these movements of the amino acid residues around the active site of chymotrypsin are indeed the cause of the change in the optical rotatory properties between 220 and 240 nm was shown by the following conformational experiments (*79*, *80*). Raising the pH from neutrality to 10, gradually transformed the CD spectrum of active chymotrypsin to one identical with that of chymotrypsinogen (see Fig. 8a). This change occurs with an apparent pK of 8.5, in agreement with the earlier observation that a change in specific rotation at 313 nm also occurs with an increase in pH and can be accounted for by the ionization of a single group with an apparent pK of 8.5 (*85*). Hess and co-workers (*79*, *80*) identified the group involved in the optical rotatory change in the following way. The amino groups of chymotrypsinogen were fully acetylated and the product was activated by trypsin to yield fully active acetylated δ-chymotrypsin. This product must have a single amino group, that of Ile 16. Circular dichroism experiments were then carried out as before, and the results are shown in Fig. 8a. The spectrum of active acetylated δ-chymotrypsin at neutral pH is identical with that of the normal enzyme at the same conditions; that of acetylated chymotrypsinogen, whether at neutral or alkaline pH, is the same as that of the unmodified precursor. An increase in pH shifted the CD spectrum of the acetylated enzyme to that of the zymogen. Further acetylation of the amino group of Ile 16 in acetylated δ-chromotrypsin also displaced the CD spectrum toward that of the zymogen even at neutral pH, as shown in Fig. 8a. These experiments clearly demonstrate the participation of Ile 16 in the observed shifts of the CD band; protonation of its α-amino group is required for the spectrum typical of the active enzyme. Modification of Ser 195 by formation of DIP-chymotrypsin (*79*) resulted in CD spectra which were identical with that of the active enzyme whether measured

83. P. B. Sigler, D. M. Blow, B. W. Matthews, and R. Henderson, *JMB* **35**, 143 (1968).
84. C. Tanford and J. G. Kirkwood, *JACS* **79**, 5333 (1957).
85. H. L. Oppenheimer, B. Labouesse, and G. P. Hess, *JBC* **241**, 2720 (1966).

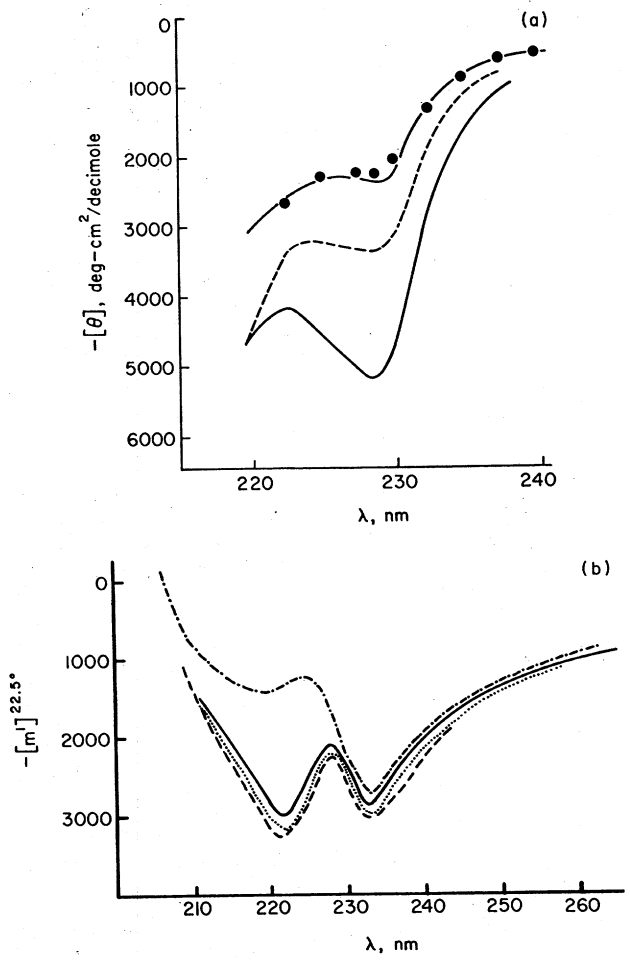


FIG. 8. (a) Circular dichroism spectra of acetylated derivatives of chymotrypsin. Lower curve, acetylated δ-chymotrypsin at pH 6.7; middle curve, acetylated δ-chymotrypsin at pH 10; upper curve, acetylated chymotrypsinogen in the pH region 6.7–10; (●) reacetylated acetylated δ-chymotrypsin in the pH region 6.7–10. (b) Ultraviolet optical rotatory dispersion curves for acetylated chymotrypsinogen and acetylated δ-chymotrypsin at pH 7.0 and pH 10.5: (–·–) acetylated δ-chymotrypsin at pH 7.0; (——) acetylated chymotrypsinogen at pH 7.0; (···) acetylated δ-chymotrypsin at pH 10.5; (---) acetylated chymotrypsinogen at pH 10.5.

at neutral or alkaline pH; this suggested that the presence of the bulky group on Ser 195 prevents the conformational change accompanied by the break of the ion pair, by steric interference with the motion of Asp 194 into solvent. The presence of the charged carboxyl inside the protein

molecule requires the continued forced protonation of the Ile 16 α-amino group even above pH 10. This was demonstrated to be true indeed by titration studies which indicate that in DIP-chymotrypsin one less group is deprotonated at high pH than in the parent enzyme (*85*).

The chymotrypsin studies are probably the best example available at present of the manner in which CD may be used as a probe of conformational changes which accompany the activation of the zymogen to an enzyme, as well as changes in solvent properties (here variation of pH). It should be stressed, however, that, while some of the side chains involved in the conformational transition have been identified, the actual nature of the electronic transitions affected is not known, nor are the details of the conformational changes known on the atomic level. The CD spectral changes observed could conceivably reflect changes in the conformations of the amino acid residues identified as being involved in the interactions; in particular, a histidine residue may undergo a spectral transition within the frequency range in question (*45*); the exact position and amplitude of its CD band may be affected by changes in charge environment, resulting from the motions of Ile 16 and Asp 194. On the other hand, it is quite possible that the spectral details between 220 and 235 nm reflect the transition of a tyrosine residue (*86*). This transition could be affected by changes in the polarity or charge distribution of the environment as the active site residues change their coordinates during activation. Indeed, it is quite suggestive that activation of chymotrypsinogen to chymotrypsin generates a tyrosine difference spectrum (*87*), indicating a change in the environment of these residues; furthermore, only three tyrosine residues are available to acetylation with *N*-acetylimidazole in chymotrypsinogen, while all four residues are accessible in α-chymotrypsin (*71*); this difference by one accessible tyrosine residue is maintained when cyanuric fluoride is used as the probe; α-, γ-, and δ-chymotrypsins have maximal reactivities of three residues with this reagent (*72, 88*), while only two groups can be induced to react in chymotrypsinogen (*71*).

A further complication in the identification of the transition in question stems from ignorance of whether the CD spectral changes between 220 and 240 nm are related to a negative band centered around 230 nm, a positive band centered at about 220 nm, or both. This results from the superposition of these weak bands on a rapidly changing negative band located at lower wavelengths. It is possible to seek an answer to this

86. M. N. Pflumm and S. Beychok, *JBC* **244**, 3973 (1969).
87. P. Benmouyal and C. G. Trowbridge, *ABB* **115**, 67 (1966).
88. Y. Hachimori, K. Kurihara, H. Horimishi, A. Matsushima, and K. Shibata, *BBA* **105**, 167 (1965).

question in the comparison of the CD and ORD spectra, which must be related by the Kronig–Kramers transform. The ORD curves of acetylated chymotrypsinogen and δ-chymotrypsin at conditions identical with those of Fig. 8a are shown in Fig. 8b (*79*). The ORD curves are identical above 230 nm; they display a trough of equal amplitude at 233 nm. Below 230 nm, a peak appears at 228 nm and a second trough at 222 nm. In the active form of the enzyme, the rotation between 230 and 210 nm is less negative than in the inactive conformation. Comparison between the presently available ORD and CD data, however, does not permit us to arrive at an unequivocal assignment of the sign, position, and intensity of this very interesting band.

2. *Ribonuclease*

The difficulties and uncertainties encountered in CD band assignment and the methods which must be employed are extremely well illustrated by the case of ribonuclease. This task would appear, *a priori*, to be quite simple since this enzyme is devoid of tryptophan residues. Yet, even in this case, it required extensive experiments to arrive at correct band assignments. The CD spectrum of ribonuclease A is shown in Figs. 4 and 9. In the far ultraviolet region it is characterized by negative absorption between 197 and 235 nm, with a maximum at 210 nm and a shoulder at 217 nm. Above 235 nm, a positive band appears at 240 nm, followed by negative absorption between 250 and 300 nm, with a maximum at 275 nm.

For purposes of conformational analysis, the spectral regions above and below 235 nm should be discussed separately. Pflumm and Beychok (*86*) decomposed the far ultraviolet spectral region into contributions from α-helical, antiparallel β, and random conformations; they used as standards spectra of poly-L-glutamic acid, poly-L-lysine, and poly-L-glutamate. They were able to fit the experimental spectrum in terms of a conformational composition of 11.5% α helix, 33% β structure, and 39% random coil, with the remainder being unassigned. This is in good agreement with the fit found by Greenfield and Fasman (*61*). In these calculations, they could keep the polypeptide band positions, half-widths and maximum amplitudes for the 222 and 207 nm α-helical bands and the 217 and 195 nm β-structure bands. Adjustments had to be made in the position (3 nm red shift) and intensity (about 50% of expected) of the 192-nm α-helical band and in the intensity (about 70% of expected) of the 198-nm random coil band. A small positive band at 226 nm had to be included to make a reasonable fit. Pflumm and Beychok (*86*) proposed as a possible explanation for the band adjustments the presence of short

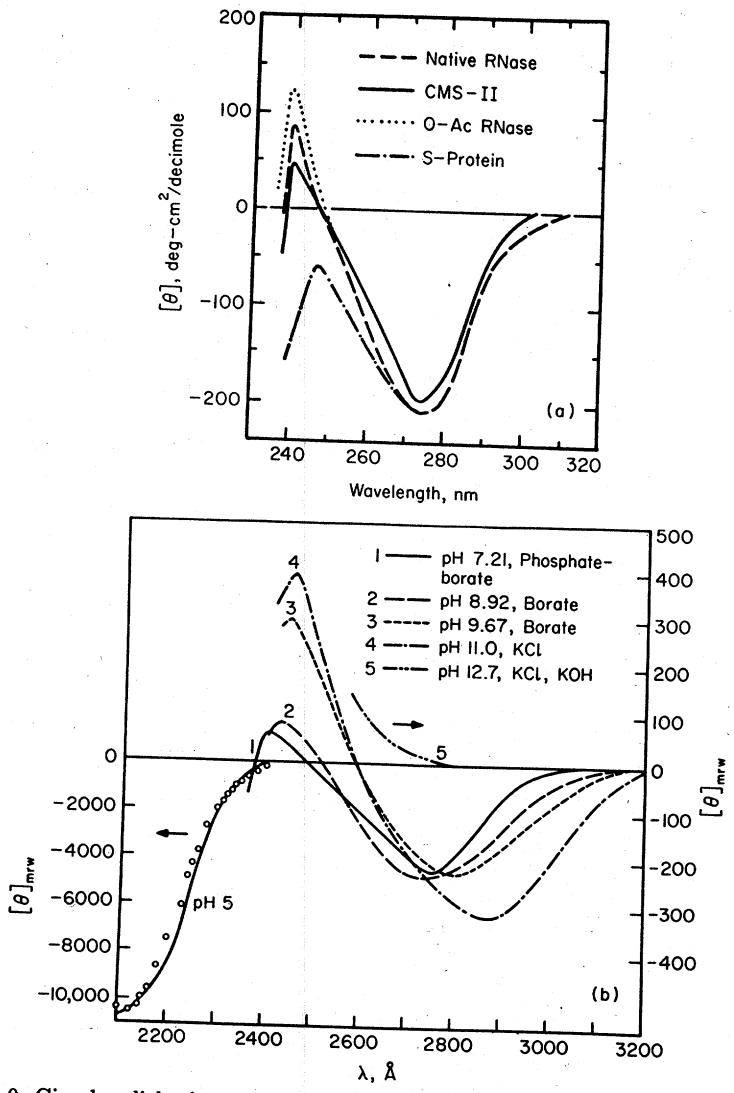


FIG. 9. Circular dichroism spectra of ribonuclease in the near ultraviolet region: (a) Ribonuclease A and various derivatives (*86, 92, 95*); (b) ribonuclease A at various pH values ($[\theta]_{mrw}$ is the ellipticity per mean residue weight) [from Pflumm and Beychok (*95*)].

or distorted helices and the observation that ribonuclease, which had been reoxidized in urea and is unordered, also yields an ellipticity value at 198 nm which is about two-thirds of that exhibited by the randomly coiled polymers. The positive band at 226 nm has been attributed to tyrosine

transitions. An alternate interpretation of the spectrum has been proposed by Schellman and Lowe (*89*). They suggest that the CD spectrum between 200 and 235 nm can be accounted for in terms of a combination of β structure and α helix, if the α-helical Cotton effect in ribonuclease is blue shifted by 5 nm. This would require a change in the exciton energy of the helical n,π^* transition of about 3 kcal/mole of residues, a situation which they find reasonable for proteins which contain short and/or distorted α helices. A contribution from tyrosine transitions in the region between 215 and 230 nm was considered unlikely by Schellman and Lowe since titration of ribonuclease to pH 11.3 has no effect on the CD in that wavelength interval. Pflumm and Beychok (*86*), however, point out that in the case of *N*-acetyltyrosineamide, the intensities of the CD spectra at pH 7 and 12 do not differ sufficiently between 215 and 230 nm to be detectable in a protein such as ribonuclease. With the elimination of this argument against the Pflumm and Beychok band assignment at 226 nm, it would seem that their conformational analysis is reasonable, in particular, since it is consistent with the findings of X-ray diffraction studies on ribonuclease (*17*, *18*). One last comment seems in order. In fitting the experimental data, Pflumm and Beychok had to assign insufficient contents of random coil conformation, leaving about 18% of the structure unaccounted for. It must be repeated again that the unordered portion of a protein molecule is not equivalent to a polymer coil in the "random flight" sense, but it is highly constrained and has little freedom of motion. Thus, it would be surprising if its CD spectrum were to average out to that of a truly randomly coiled polypeptide chain, and ribonuclease seems to be a good case in point.

Let us turn now to the structural information inherent in the positive band at 240 nm. The assignment of this band has been the subject of several studies (*65*, *86*, *90–92*). It can result from either a tyrosyl or a disulfide transition. Chemical modification and variation of pH permitted assigning this band to a tyrosine transiton. The evidence in favor of this assignment is manyfold: Pflumm and Beychok (*86*) have found that the intensity increases and the band is red shifted when the pH is raised from 8.9 to 11, as would be expected from tyrosine ionization; the intensity at 240 nm increases upon acetylation of the three accessible tyrosines as shown in Fig. 9a; nitration with tetranitromethane results in diminished intensity. Simons and Blout (*92*) have examined the pH dependence of this band as well as the differences in the CD spectra of

89. J. A. Schellman and M. J. Lowe, *JACS* **90**, 1070 (1968).
90. R. J. Simpson and B. L. Vallee, *Biochemistry* **5**, 2531 (1966).
91. N. S. Simmons and A. N. Glazer, *JACS* **89**, 5040 (1967).
92. E. R. Simons and E. R. Blout, *JBC* **243**, 218 (1968).

ribonuclease S, the S protein and ribonuclease S reconstituted by addition of the S peptide to the S protein. Acid denaturation eliminates this band in ribonuclease A at pH 2 and in ribonuclease S at pH 3.5. As shown on Fig. 9a, the S protein does not have this positive band; this derivative differs from the parent enzyme in that it contains four available tyrosines rather than three. This evidence again implicates a tyrosine residue as being responsible for the 240-nm band and specifically the group whose environment changes on removal of the S peptide, i.e., Tyr 25. The chemical modification data (*86*), on the other hand, point to the participation of a solvent accessible tyrosine. It would appear, therefore, that the band observed at 240 nm contains contributions from transitions of both at least one accessible and one inaccessible tyrosine residues of ribonuclease.

The 240-nm band of ribonuclease has also been found to be an extremely sensitive probe of minor conformational changes. Following the discovery by Anfinsen and co-workers (*93*, *94*) that when ribonuclease is reduced and reoxidized its enzymic activity is essentially regained, Pflumm and Beychok carried out a detailed investigation of this process by CD (*95*). They found that the CD spectrum of the reoxidized enzyme differs from that of the native enzyme in a decrease of positive intensity at 240 nm. Fractionation on a column resulted in several components, one of which, CMS-II, exhibited full enzymic activity. Its CD spectrum, shown in Fig. 9a, however, differed again from that of the native enzyme in the 240-nm region, while it was essentially identical with that of the parent enzyme at other wavelengths. Thus, it appears that an enzyme, ribonuclease, may have two fully active forms which differ in small conformational features, the exact nature of which is not yet known.

Another interesting use of this band has been made by Simons *et al.* (*96*) who found that when ribonuclease A was heated at pH 6.46, the intensity at 240 nm decreased in a bimodal fashion as shown in Fig. 10. The first change is gradual and noncooperative; it is followed by a cooperative transition which corresponds to the thermal denaturation of the enzyme. It would appear, therefore, that when ribonuclease is heated from 15° to 50°C, a small local change in conformation occurs. Comparison with similar data on the S protein, which does not display this bimodality, and with the above-described observations, has led the authors to speculate that the first slow change in ellipticity corresponds

93. C. B. Anfinsen and E. Haber, *JBC* **236**, 1361 (1961).
94. C. J. Epstein, R. F. Goldberger, D. M. Young, and C. B. Anfinsen, *ABB* Suppl. 1, 223 (1962).
95. M. N. Pflumm and S. Beychok, *JBC* **244**, 3982 (1969).
96. E. R. Simons, E. G. Schneider, and E. R. Blout, *JBC* **244**, 4023 (1969).

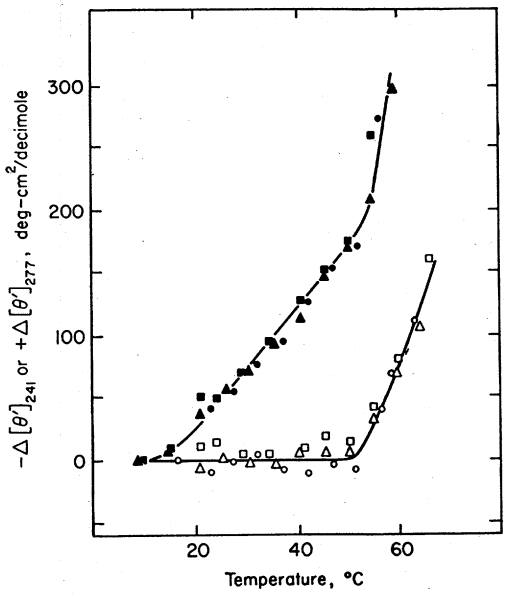


FIG. 10. Molar ellipticity change of RNase A as a function of temperature: (■) 0.1 *M* phosphate at 241 nm, (□) 277 nm; (●) 0.1 *M* acetate at 241 nm, (○) 277 nm; (▲) 0.1 *M* sulfate at 241 nm, and (△) 277 nm [from Simons and Blout (*92*)].

to an initial detachment of noncovalent bonds between the S-peptide and S-protein portions of the enzyme, with the release to solvent of one of the abnormal tyrosines. It is particularly interesting that the 275-nm band, which will be discussed now, does not undergo the first transition but changes sharply only in the general unfolding of the secondary structure. It would appear reasonable to infer from this that Tyr 25 makes no contribution to the higher wavelength band and that variations in the two bands may serve as probes of two distinct conformational changes.

Assignment of the negative band at 275 nm again is not immediate. In the absence of tryptophan, this band may result from either tyrosine or cystine transitions (*97*). Simpson and Vallee (*90*) and Beychok (*65*) found that when pH is raised to 11, the band is red shifted to 288 nm and its ellipticity increases by 50%, as shown in Fig. 9b. This suggests the participation of exposed and ionizable tyrosines. Acetylation of tyrosine hydroxyl groups, however, results in only a 10% decrease in band intensity, implicating either buried tyrosines or disulfides as

responsible for the remainder of the band. That disulfides do make a contribution is supported by the observation that a small negative CD band persists at 275 nm even in 8 *M* urea at pH 11, conditions at which tyrosines should be totally randomized (*86*). Nitration experiments cast further light on the nature of the groups involved. Beaven and Gratzer (*98*) found that nitration of 1.8 tyrosines/mole of protein resulted in the appearance of a dichroic band at 360 nm with no changes in intensity at 275 nm, suggesting that the new band at 360 nm results from previously optically inactive exposed residues. Nitration of all three exposed groups (*95*) is accompanied by a decrease in optical activity at 275 nm, as well as in the far ultraviolet, indicating some conformational change. It would appear, therefore, that the 275 nm band stems from the summation of transitions of disulfides, some buried tyrosines, and possibly one or two of the exposed tyrosines.

Summarizing all the available information on the behavior of the 240- and 275-nm CD bands leads to the conclusion that both the exposed and buried tyrosines of ribonuclease are in different environments. Thus, Tyr 25 seems to be optically inactive, while the other buried residues contribute to the CD; on the other hand, only the least accessible of the three exposed residues seems to be optically active since nitration of two groups does not change the spectrum at 275 nm; that one of the exposed groups is present in asymmetric environment is required by the observation of the pH dependence of the 275-nm band below the onset of irreversible changes. Further support for this conclusion may be drawn from solvent perturbation spectroscopy (*99*) and chemical modification studies (*100*). Both studies indicate that one of the exposed residues is significantly less accessible to the environment than the other two.

3. *Carbonic Anhydrase*

The third specific system which we shall describe, that of carbonic anhydrase, will serve to illustrate two problems. First, it is probably the enzyme with the most complicated CD spectrum that has been subjected to detailed analysis; second, it has been the object of a detailed study of the effect of binding of an inhibitor on the CD spectrum.

The CD spectra of a number of isozymes of carbonic anhydrase from various sources have been reported by several authors (*12*, *13*, *23*, *46*, *101*). All are similar in their general complexity; the small differences in

98. G. H. Beaven and W. B. Gratzer, *BBA* **168**, 456 (1968).
99. T. T. Herskovits and M. Laskowski, Jr., *JBC* **235**, PC56 (1960).
100. M. J. Gorbunoff, *Biochemistry* **6**, 1606 (1967).
101. M. J. Gorbunoff, *ABB* **138**, 684 (1970).

their amino acid compositions, however, do result in significant varia
tions. This suggests that the various enzymes—whether human, bovin
or monkey—have similar general structural features but differ mainl
in details of tertiary structure. The CD spectra of human carbonic a
hydrase B and monkey enzyme C at pH 7.5 are shown in Fig. 11. Th
positions of the various extrema are indicated in the figure. Beycho
et al. (*23*) carried out a detailed study of the pH dependence of th
ORD and CD of the human enzymes, while Rosenberg (*28*) reporte

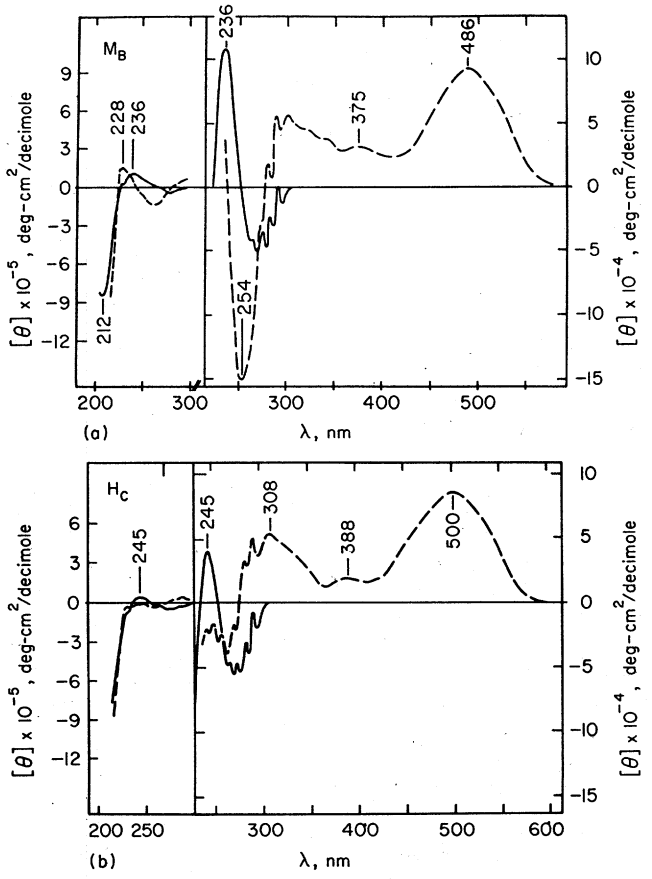
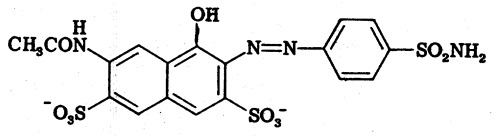


FIG. 11. Circular dichroism spectra of (a) *Macaca mulatta* carbonic anhydrase
(M_B) and (b) human carbonic anhydrase C (H_C) and their 1:1 complexes wi
Neoprontosil. (——) Isozyme and (- - -) isozyme plus equimolar Neoprontosil. A
solutions contained $5 \times 10^{-5} M$ enzyme ($+5 \times 10^{-5} M$ azosulfonamide in the case
the complexes), 0.025 M tris, pH 7.5, 25°. The ellipticities are calculated p
decimole of protein, rather than mean amino acid residue [from Coleman (*46*)].

similar ORD results for the human and bovine species. It was found that the extrema located at 275 and 236 nm shift to higher wavelengths when the pH is raised to above 10, suggesting that those bands correspond to tyrosine transitions. The higher wavelength extrema are certainly due to tryptophans (*70*); carbonic anhydrase contains no disulfide bridges. In the lower wavelength region, the spectra are difficult to interpret. They are in general weak and the band positions do not correspond to those of any given ordered structure, such as the α helix or β conformation. In this spectral region, as well, it appears that aromatic side chain transitions predominate. Beychok *et al.* (*23*) have examined the effects on CD of both acid and alkaline denaturation. In both cases, many of the spectral details, in particular, in the region between 250 and 300 nm, disappear suggesting the loosening of enzyme structure, with a strong enhancement of the freedom of rotation of the aromatic residues (*70*). The CD and ORD spectra, in the region below 240 nm, would suggest an increase in the α-helical contents on acid denaturation, in agreement with the infrared spectra (*11*). The ORD spectra at alkaline pH values are consistent with the presence of significant amounts of α-helical structure. Since the infrared spectra of the bovine enzyme at neutral pH and at pH 12 are essentially identical, one might speculate that the high pH CD and ORD spectra describe a secondary structure not very different from that of the native enzyme. X-ray crystallographic studies at 5.5 Å resolution (*102*) indicate that human carbonic anhydrase C may contain as much as 30% α helix; examination of the manner in which the polypeptide chain is folded into the globular structure indicates some regions in which the chains run parallel to each other, suggesting the presence of β conformation.

Coleman (*46*) has carried out a particularly interesting CD study on the interactions of various carbonic anhydrases with a nonoptically active inhibitor, 4′-sulfamylphenyl-2-azo-7-acetamido-1-hydroxynaph-



(I)

thalene-3,6-disulfonate (Neoprontosil). This compound is known to complex with the various carbonic anhydrases and to inhibit their activity.

102. K. Fridborg, K. K. Kannan, A. Liljas, J. Lundkin, B. Strandberg, R. Strandberg, B. Tilander, and G. Wirén, *JMB* **25**, 505 (1967).

X-ray studies have shown that the inhibitor is inserted into a crevice leading to the zinc atom (*102*).

The CD spectra of monkey carbonic anhydrase B and human enzyme C in the free and complexed states are shown in Fig. 11. (The spectra of the human and monkey enzymes C are essentially identical.) It is evident that complexing to the enzyme of Neoprontosil, which by itself is devoid of optical activity, results in dramatic CD spectral changes. It is particularly striking that the positions of the new bands are different for the two enzymes, which are very similar in amino acid composition. This suggests that there are considerable structural differences around the active sites of the two isozymes; particularly striking is the presence of a large negative band at 254 nm in the monkey carbonic anhydrase B complex and its absence in human carbonic anhydrase C complex. These structural differences have been found by Coleman (*46*) to be paralleled by differences in the esterase activities of the enzymes. Therefore, it appears that the azosulfonamide acts as a highly sensitive probe of the active-site topography.

The azosulfonamide is optically inactive. Its combination with the protein, however, induces strong optical rotatory power which is of a magnitude typical of inherently dissymmetric chromophores. It seems, therefore, that the inhibitor becomes highly immobilized in an asymmetric environment within the protein molecule. Coleman (*46*) concludes that the induced optical activity must result from coupling with transitions of the protein or from dissymmetric electrostatic perturbations produced by charged groups or dipoles of the protein. In fact, the observed CD spectral shifts are consistent with the location of this molecule within a hydrophobic cavity (*103*, *104*) in the active center of carbonic anhydrase.

The extreme complexity of overlapping transitions which result from the interaction of the inhibitor with the enzyme is demonstrated in Fig. 12. Here the CD spectrum between 235 and 500 nm of the bovine enzyme B and its complex have been decomposed into sets of Gaussian bands. Very strikingly, seven bands are needed to account for the enzyme spectrum, while an additional six bands arise on binding of the inhibitor. A particularly interesting feature is that four weak bands in the aromatic region of the enzyme spectrum (at 267, 275, 283, and 296 nm) are found intact in the spectrum of the complex. Therefore, the groups responsible for these transitions remain unaffected by the presence of the sulfonamide. These bands correspond to both tyrosine and tryptophan

103. M. E. Riepe and J. H. Wang, *JACS* **89**, 4229 (1967).
104. R. F. Chen and J. C. Kernohan, *JBC* **242**, 5813 (1967).

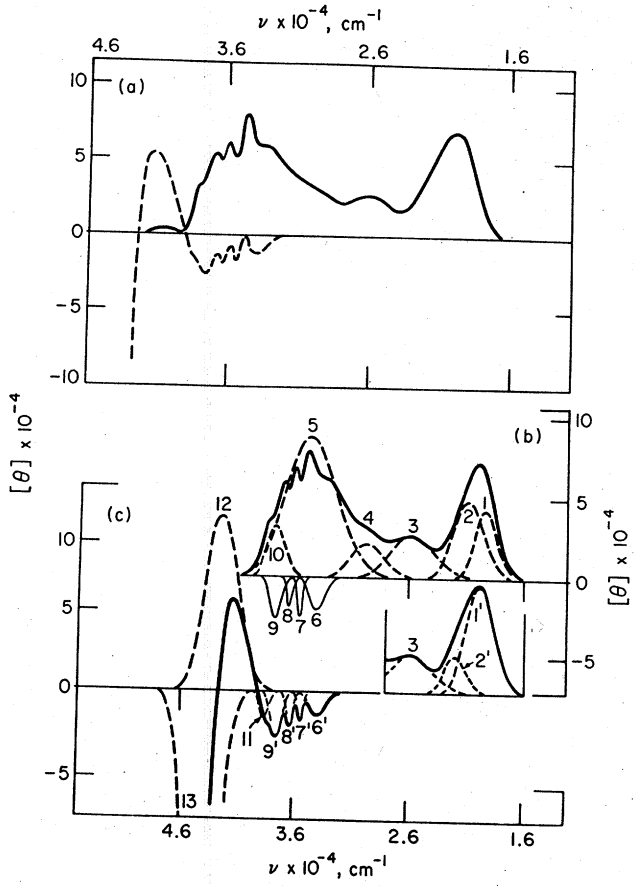


FIG. 12. Circular dichroism spectra of bovine carbonic anhydrase B and its 1:1 complex with Neoprontosil. Resolution into a set of overlapping Gaussian ellipticity bands: (a) (——) observed CD spectrum of the complex; (- - -) observed CD spectrum of the native enzyme. (b) (complex) (——) Resultant envelope for the ten Gaussian bands labeled from 1 to 10; (---) bands attributable to the sulfonamide; (——) bands attributable to the protein. The subset curve, bands 1′ and 2′, shows an alternate fitting of the lowest energy region. (c) (native enzyme) (——) Resultant envelope for the seven Gaussian bands labeled on the figure. In (b) and (c), the envelopes correspond within the limit of error to the experimental CD curves. The ellipticities are calculated per decimole of protein [from Coleman (*46*)].

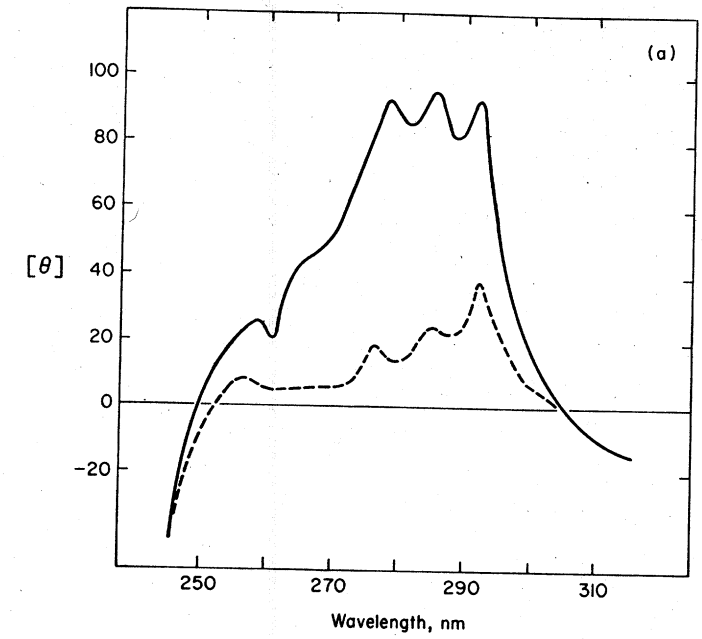
transitions which, by inference, should be located in a region of the enzyme that is distant from the active center. A further observation of great interest is the fact that the CD spectra of the enzyme–inhibitor complexes are different whether the metal atom on the active site is Zn(II) or Co(II) (*46*). This may result from differences in binding

affinity or from slight conformational differences in the active center in the presence of the different metals.

4. *Pyrocatechase*

An example of the effect of a bound metal atom on the CD spectrum of an enzyme is found in the recent study on pyrocatechase (*105*). Pyrocatechase is an enzyme which requires ferric iron for enzymic activity. Nakazawa *et al.* (*105*) examined the effects of iron binding and interaction with substrate on the CD spectrum of this enzyme, with pertinent results shown in Fig. 13. It is very striking that in both cases the qualitative features of the spectrum in the aromatic region remain essentially unchanged but the band intensities change drastically. The spectrum in the near ultraviolet is positive with five maxima at 292, 285, 278, 265, and 255 nm. Removal of iron causes a decrease in all the bands; addition of catechol results in a decrease in band intensity above 265 nm and an enhancement in positive ellipticity below that wavelength. While removal of iron seems to affect equivalently all the aromatic transitions without any changes in secondary structure, binding of the substrate is reflected differently in the bands typical of tryptophan (285 and 292 nm) and those characteristic of the tyrosine phenolic ring. Thus, when substrate is bound, positive CD extrema appear at 253 and 275 nm; these are known to be related to tyrosines, and in the case of this particular enzyme the 255-nm band has been unequivocally related to a phenolic transition. Catechol itself is optically inactive (*105*). It is interesting to speculate whether its immobilization in the active site of the enzyme does not impart to it optical activity, as a result of effects similar to those found with carbonic anhydrase. The iron enzyme also displays dichroism in the visible region, with a negative maximum at 327 nm and a broad negative band centered at 500 nm. Removal of iron eliminates these bands totally with no changes in secondary structure. When the iron-containing enzyme was heated, the ellipticities at 500, 327, 292, and 285 nm changed linearly with a decrease in enzymic activity, again without any changes in secondary structure, as evidenced by a lack of change of the CD spectrum below 235 nm. In the absence of iron, no effect could be detected upon addition of the substrate. It would appear, therefore, that the binding both of the metal ion and of the substrate induce subtle conformational changes in the active site which are directly related to the enzymic activity. In neither case is a major change in secondary structure necessary.

105. A. Nakazawa, T. Nakazawa, S. Kotani, M. Nozaki, and O. Hayashi, *JBC* **244**, 1527 (1969).



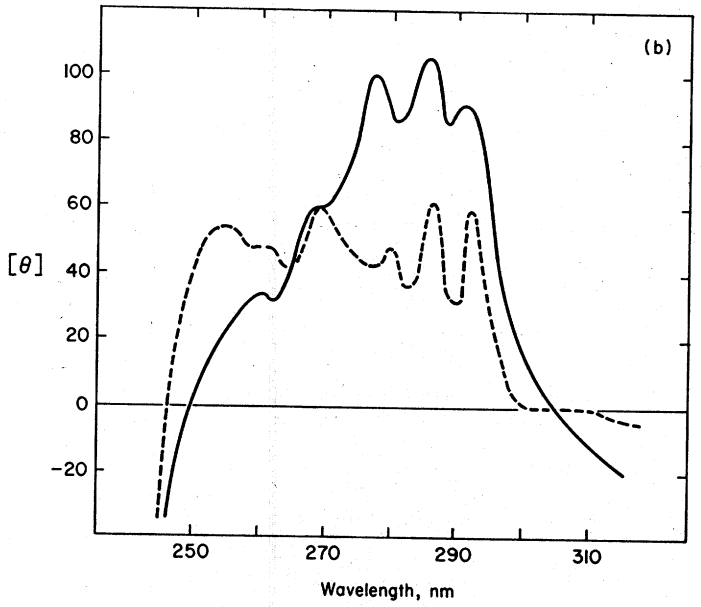


FIG. 13. (a) Circular dichroism of the holo- (——) and apopyrocatechase (---) in 0.05 *M* tris Cl, pH 8.0 [from Nakazawa *et al.* (*105*)]. (b) The effect of pH on the ultraviolet circular dichroism of pyrocatechase: (——) before the addition of catechol; (---) after the addition of catechol [from Nakazawa *et al.* (*105*)].

extent to which this technique is capable at present of probing the structure of enzymes in solution. It is evident that, while much qualitative information of a general nature may be obtained, little actual structural information follows. At best, it is possible to determine which types of residues in the three-dimensional structure of the enzyme are close to the active site or are affected by interaction of the enzyme with substrate or inhibitor as well as by changes in conformation. In all cases, where more detailed conclusions could be drawn, information had to be drawn from other sources. For example, the detailed interpretation of structural changes responsible for the changes in the CD spectrum between 220 and 235 nm when chymotrypsinogen is activated to chymotrypsin could certainly not have been carried out on the basis of CD data alone. In fact, CD turned out to be an excellent probe for following the changes; these experiments, however, were done in the blind from the point of view of CD since the exact nature of the transitions followed is still unknown.

VI. Ultraviolet Difference Spectroscopy

Because of the strong overlapping of bands ultraviolet absorption spectroscopy of proteins in solution can yield only gross general structural information. A much more sensitive way of detecting small discrete changes in the environment of particular absorbing groups is afforded by the differential technique. In this approach, two solutions of the same protein in different environments are compared directly at identical concentrations; since one solution serves as the sample and the other as the blank, all the common features of their spectra cancel out and only those transitions which had been displaced with respect to each other because of alterations in the environment are manifested by positive or negative differential bands. This enzyme structural probe is based on the fact that the transition moments which give rise to the absorption bands of chromophores are strong functions of their interactions with the environment. Electrical transition moments may be perturbed by a number of factors; these include the polarity of the environment, the polarizability of the solvent, the presence of charges or dipoles in the vicinity

of the chromophore, and the formation of hydrogen bonds (for example, in the case of the tyrosine phenolic radical). Such interactions lead to shifts both in the positions and intensities of the bands. For example, Yanari and Bovey (*106*) have shown that the spectra of indole, phenol, and benzene undergo a red shift when the refractive index of the solvent is increased. In general, transfer of aromatic side chains from an aqueous to a hydrophobic medium results in a red shift and an exaltation of the absorption. The general principles of difference spectroscopy and the types of information that can be obtained have been adequately reviewed (*107–109a*) and will not be discussed in detail here.

Ultraviolet difference spectroscopy of enzymes has gained its widest applications in studies aimed at the probing of general conformational changes which occur, for example, when the pH is varied over a wide range (acid difference spectra), and in investigations of the dissociation behavior of ionizable chromophores, in particular of tyrosines. While the first type of application is outside the scope of this chapter, the second will be discussed in a later section. Suffice it only to demonstrate here by a few specific examples the type and degree of insight that may be obtained into changes in the environment of particular chromophoric groups which occur when a zymogen is activated to an enzyme or when the enzyme interacts with ligands such as substrates or inhibitors.

The specific residues which have been used most extensively as markers of conformational details are tryptophans and tyrosines. When their environment is perturbed, these residues yield difference absorption bands in the near ultraviolet region between 270 and 300 nm. Specifically, the unionized tyrosine difference absorption bands occur at about 278 and 287 nm, while the tryptophan bands are at about 284 and 292 nm, with possibly a weak band close to 275 nm (*107, 110, 111*). Thus, below the pH of tyrosine ionization, the presence of a difference band at 292 nm indicates the involvement of tryptophans, while similar bands at lower wavelengths may result from either tyrosines or tryptophans and require for their complete interpretation independent information such as knowledge of the exact amino acid composition or of the manner in which the difference spectra are affected by chemical modifications.

106. S. Yanari and F. A. Bovey, *JBC* **235**, 2818 (1960).
107. D. B. Wetlaufer, *Advan. Protein Chem.* **17**, 303 (1962).
108. M. Laskowski, Jr., *Federation Proc.* **25**, 20 (1966).
109. T. T. Herskovits, "Methods in Enzymology," Vol. 11, p. 748, 1967.
109a. J. W. Donovan, *in* "Physical Principles and Techniques of Protein Chemistry" (S. J. Leach, ed.), Vol. 1, p. 102. Academic Press, New York, 1969.
110. E. J. Williams and M. Laskowski, Jr., *JBC* **240**, 3580 (1965).
111. T. T. Herskovits and Sr. M. Sorensen, *Biochemistry* **7**, 2523 (1968).

A. Solvent Perturbation Spectroscopy

A very ingenious approach to the detailed probing of enzyme topography is that of solvent perturbation difference spectroscopy developed by Laskowski and his school (*99, 108, 110, 112, 113*). The method is based on the very simple principle that spectral bands undergo small shifts when the polarity of the environment is changed. Thus, if an absorbing group is present in the surface of the enzyme in contact with aqueous solvent, addition of a nonaqueous component to the solution results in slight alterations in its spectrum since its transition moments are affected by interactions with the new solvent component. To the contrary, if the same group is present inside the hydrophobic interior of the protein molecule, addition of the new solvent component, if it does not alter the conformation of the protein, should not affect its absorption spectrum. Operationally, a difference spectrum is measured between a solution of the protein in aqueous medium containing 20% of the inert perturbant (e.g., ethylene glycol, glycerol, sucrose, methanol, and polyethylene glycol) and the same solution without the perturbant. A difference spectrum such as the one shown in Fig. 14 is obtained (*114*). The existence of such a difference spectrum indicates that absorbing groups are in sufficient contact with solvent for their transition moments to be perturbed by the additive. The extent of group exposure is then calculated from the ratio of the differential peak intensities with those obtained with a fully unfolded protein. For example, the 292-nm peak present in the chymotrypsinogen difference spectrum, when polyethylene glycol is used as perturbant, has a height ($\Delta\epsilon/\epsilon_{282}$) of 0.026 and that of the hypothetical denatured protein in water has a height of 0.063 (*114*). Since the last value corresponds to perturbation of all absorbing groups, i.e., contact of all groups with solvent, the ratio of these two numbers yields the extent of exposure of the pertinent groups, namely, $0.026/0.063 = 0.41$. Since the peak at 292 nm corresponds to tryptophan residues (*110*), this means that chymotrypsinogen at pH 4.4 has 41% of its tryptophans exposed to solvent if contact with polyethylene glycol is used as criterion.

If perturbants of different sizes are used, this technique becomes a probe of the surface topography of the enzyme molecules. Laskowski

112. M. Laskowski, Jr., R. H. Cramer, T. T. Herskovits, and C. C. Wang, *Federation Proc.* **19**, 343 (1960).

113. T. T. Herskovits and M. Laskowski, Jr., *JBC* **237**, 2481 (1962).

114. E. J. Williams, T. T. Herskovits, and M. Laskowski, Jr., *JBC* **240**, 3574 (1965).

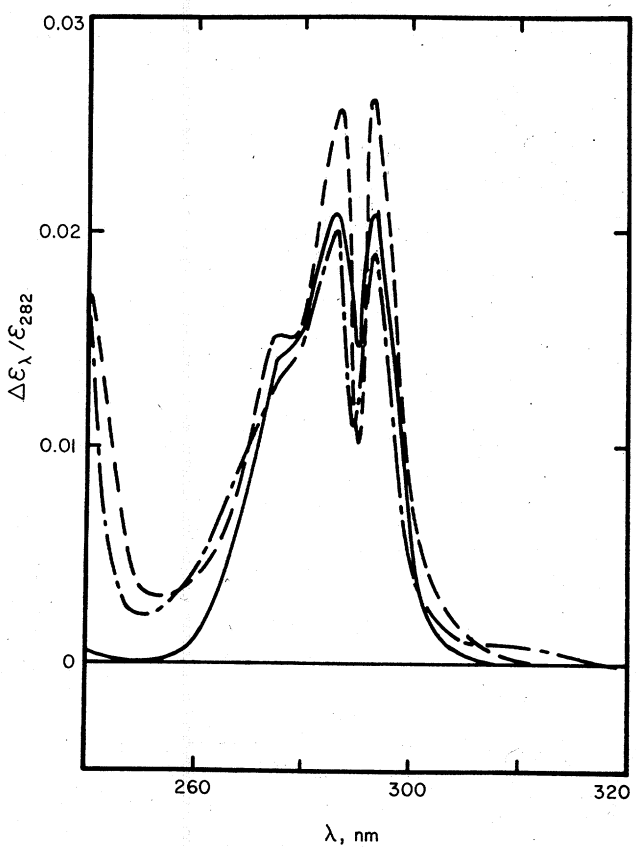


FIG. 14. The solvent perturbation difference spectra of chymotrypsinogen (---), α-chymotrypsin (——), and diisopropylphosphoryl-α-chymotrypsin (-——-) in 0.05 *M* sodium acetate–acetic acid buffer, pH 4.4, 0.1 *M* NaCl. The perturbant is 20% (v/v) polyethylene glycol; protein concentration = 0.13% [from Williams *et al.* (*114*)].

(*108*) has discussed two distinct situations that must be considered. The first concerns the presence of crevices on the protein surface, the second deals with partially exposed groups. Crevices may be detected by using perturbants with various van der Waals radii. The principle is illustrated in Fig. 15. It is seen that if some of the chromophores are located within narrow crevices such chromophores can be perturbed only by perturbants small enough to enter the crevice; they are not affected and hence remain undetected by larger ones. On the other hand, chromophores located on the surface or within wide crevices can be detected by a greater variety of perturbants of various sizes.

Another situation which must be considered is the existence of the

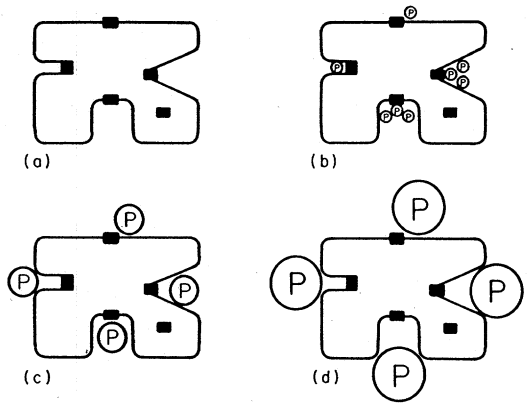


FIG. 15. Schematic diagram showing the use of perturbants of different sizes to detect crevices within proteins. The black rectangles denote chromophores; the circles, P, denote perturbant molecules. (a) Reference protein solution; (b) smallest perturbant approaches all four exposed residues, giving 80% exposure; (c) intermediate size perturbant approaches three residues, giving 60% exposure; and (d) large perturbant approaches one residue, giving 20% exposure.

chromophoric groups in a partially exposed state. The detection of 50% exposure of a certain type of group with a variety of perturbants may have several interpretations. This may mean that all the groups are half-exposed (e.g., with only one side in contact with solvent), that half of the groups are fully exposed and half are completely buried, or that there is a combination of degrees of exposure which average out to 50%. Resolution of this question usually requires the recourse to other experiments such as chemical modification. For example, in the case of chymotrypsinogen, oxidation of three tryptophans with *N*-bromosuccinimide eliminated the solvent perturbation difference spectrum and no further change was evident on oxidation of the remaining residues (*110*). It seems reasonable to conclude then that in chymotrypsinogen three tryptophans are fully exposed and five are completely buried. In ribonuclease, solvent perturbation indicates that about half of the tyrosine residues are exposed to solvent (*112*, *115*); chemical modification studies with cyanuric fluoride (*100*) showed that the three normally titrating residues are accessible to different extents to this reagent; in fact, two groups react quite readily, while the third group requires some loosening of the enzyme. This conclusion was confirmed by detailed solvent perturbation spectroscopy studies (*115*) in which the spectral data could be accounted for best in terms of two fully exposed and two partially buried tyrosine residues.

At present, the topography of a number of enzymes has been probed using this technique. It has been established, as a general rule, that even though they are hydrophobic in character at least part of the tyrosines and tryptophans are exposed to solvent, the values usually clustering about 50%. In more detailed studies on some enzymes it has been possible to show that the tyrosines and tryptophans of glutamic dehydrogenase (*116*) and the tryptophans of α-lactalbumin (*117*) are located within crevices. On the other hand, none of the tyrosines of ribonuclease (*112*, *115*) nor of the tryptophans of chymotrypsinogen (*114*) and lysozyme (*114*, *118*) is located within crevices. In pepsin (*119*) and aldolase (*119*, *120*) both types of residues exhibit various degrees of exposure but are not located within crevices. Other residues within enzymes that have been examined with a single perturbant only, thus precluding conclusions concerning the existence of crevices, include the tyrosines of taka-amylase (*121*), lactate dehydrogenase (*122*), glyceraldehyde-3-phosphate dehydrogenase (*122*), and human carbonic anhydrase B (*123*) and the tryptophans of carboxypeptidase (*124*), human carbonic anhydrase B (*123*) and trypsin (*125*). In the last case, the interesting observation was made that complexing of trypsin with pancreatic inhibitor results in little change in the degree of exposure of the tryptophans of either protein, indicating that these groups are removed from the site of enzyme–inhibitor complexing (*125*).

B. Typical Cases

1. *Staphylococcal Nuclease*

A particularly striking example of the effect of ligand binding on group exposure is found in staphylococcal nuclease (*126*). This enzyme contains seven tyrosines and one tryptophan. Cuatrecases *et al.* (*126*) examined its solvent perturbation difference spectra in the absence and

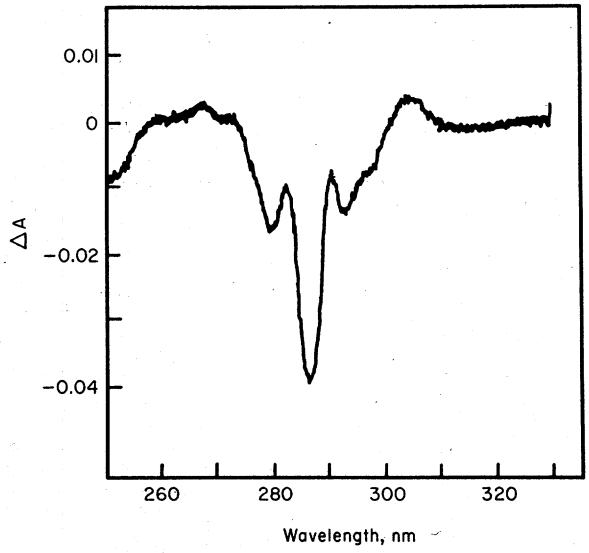
116. D. Cross and H. Fisher, *Biochemistry* **5**, 880 (1966).
117. M. J. Kronman and L. G. Holmes, *Biochemistry* **4**, 526 (1965).
118. K. Hayashi, T. Imoto, and M. Funatsu, *J. Biochem.* (*Tokyo*) **55**, 516 (1964).
119. T. T. Herskovits and Sr. M. Sorensen, *Biochemistry* **7**, 2533 (1968).
120. J. W. Donovan, *Biochemistry* **3**, 67 (1964).
121. T. Friedman and C. J. Epstein, *JBC* **242**, 5131 (1967).
122. S. Libor, E. Elodi, and Z. Nagy, *BBA* **110**, 484 (1965).
123. R. H. Stellwagen, L. Riddiford, and J. T. Edsall, *Abstr., 145th ACS Meeting, New York, 1963* p. 66C.
124. H. Fujioka and R. Imahori, *J. Biochem.* (*Tokyo*) **53**, 244 (1963).
125. H. Edelhoch and R. F. Steiner, *JBC* **240**, 2877 (1965).
126. P. Cuatrecases, S. Fuchs, and C. B. Anfinsen, *JBC* **242**, 4759 (1967).

presence of the competitive inhibitor, deoxythymidine 3′,5′-diphosphate Using glycerol and ethylene glycol as perturbants, it was found that in the free enzyme 4–5 tyrosines are exposed to solvent, while the tryptophan is completely buried. Binding of the inhibitor leads to dramatic spectral changes: Only one tyrosine remains exposed to solvent, while the inhibitor itself gives no solvent perturbation spectrum. These results indicate that attachment of the diphosphate to the active site of the enzyme causes it to become surrounded with protein and to be taken out of contact with the solvent. This must be accompanied either by a conformational change which buries the tyrosines or by direct interaction between the inhibitor and 3–4 tyrosines in such a way that the latter become shielded from contact with solvent.

2. *Chymotrypsin*

The various ramifications of difference spectroscopy have been used quite successfully to probe the structural events that accompany the zymogen → enzyme activation and enzyme–substrate interactions in the chymotrypsin system.

The activation of chymotrypsinogen to chymotrypsin generates the difference spectrum shown in Fig. 16 (*87*, *126a*). The appearance of



differential absorption bands at 292, 287, and 278 nm indicates that both tryptophan and tyrosine transitions are affected; thus, the environment of both types of residues changes on activation.

Williams *et al.* (*114*) and Oppenheimer *et al.* (*127*) have carried out solvent perturbation difference spectroscopy studies on chymotrypsinogen, α-chymotrypsin, and diisopropylphosphoryl-α-chymotrypsin. The difference spectra obtained when polyethylene glycol is used as perturbant are shown on Fig. 14. Both tyrosine and tryptophan residues are exposed to solvent in all three proteins. The results obtained with several perturbants of different sizes for the 292-nm difference band characteristic of tryptophans are summarized in Table IV. Williams *et al.* (*114*) have concluded from these data that the exposure of tryptophans is not changed significantly in the activation process. The observed decrease in exposure to large, strongly interacting perturbants, dimethyl sulfoxide and polyethylene glycol, suggests a small local conformational change. The differences between DIP-α-chymotrypsin and α-chymotrypsin are very small. They are of a sufficient magnitude, however, to suggest that subtle changes in the environment of a tryptophan residue occur on diisopropylphosphorylation of Ser 195. In fact, Oppenheimer *et al.* (*127*) concluded that the differences in the spectra of α-chymotrypsin and the DIP derivative result from a tryptophyl residue which is buried in both the enzyme and the derivative.

That the environment of tyrosines is altered on activation is evident from the activation difference spectrum (Fig. 16) (*87*), which has a

TABLE IV

RELATIVE EXPOSURE OF TRYPTOPHYL RESIDUES OF CHYMOTRYPSINOGEN, α-CHYMOTRYPSIN, AND DIISOPROPYLPHOSPHORYL-α-CHYMOTRYPSIN (*114*)

	$(\Delta\epsilon/\epsilon)_{rel}$[a]		
Perturbant 20% (v/v)	Chymotrypsinogen	α-Chymotrypsin	Diisopropylphosphoryl-α-chymotrypsin
Sucrose	0.49 (0.55)	0.47 (0.52)	0.44 (0.48)
Glycerol	0.43 (0.35)	0.40 (0.33)	0.38 (0.31)
Ethylene glycol	0.44 (0.35)	0.41 (0.33)	0.43 (0.35)
Dimethyl sulfoxide	0.39 (0.31)	0.30 (0.23)	0.28 (0.22)
Polyethylene glycol	0.41 (0.30)	0.33 (0.24)	0.30 (0.22)

[a] Exposure estimated relative to the hypothetical denatured proteins in water; values in parentheses are relative to the model mixture analogs.

strong band at 287 nm. This is supported by the difference betwee solvent perturbation difference spectra of the zymogen and the enzym (Fig. 14) (*114*), as well as by the results of chemical modification ex periments (*71*). In the case of the tyrosines, however, none of th spectral data can be interpreted directly in terms of changes in tyrosin exposure. On activation, Tyr 146 becomes the C-terminal group of th B chain. This should have two effects on the chromophore (*71*): first it should become more available to solvent and thus be immersed in an environment of different polarity; second, the appearance of a α-carboxyl group on the same residue should alter significantly th charge environment of the phenolic ring. Wetlaufer *et al.* (*128*) have shown that ionization of the α-carboxyl group of an aromatic amino acid perturbs its spectrum. The change in tyrosine absorption on removal of residues 147 and 148 may, therefore, reflect in great part the introduction of a new negative charge in the vicinity of the side chain of Tyr 146.

Difference spectroscopy has also been used to probe chymotrypsin-substrate interactions. Benmouyal and Trowbridge (*87*) determined the difference spectra between α-chymotrypsin and its complexes with several ligands. The difference spectra obtained when *p*-toluenesulfonylarginine methyl ester and acetyl phenylalanine ethyl ester were used as ligands are very complicated and quite different in character. Since neither ligand absorbs at wavelengths above 275 nm, the difference spectra must reflect changes in the environment of enzyme chromophoric residues. Since both the positions and signs of the difference bands are different for the two substrates, it would appear that their binding to the enzyme induces nonidentical local structural perturbations. Similar effects have been observed by Burr and Koshland (*129*) in experiments on the binding of substrate to α-chymotrypsin which had been chemically modified by the incorporation of a chromophoric reporter group (see Chapter 7 by Koshland, Volume I).

An elegant application of solvent perturbation spectroscopy to enzyme–substrate interaction is the study of Mercouroff and Hess (*130*) on *trans*-cinnamoyl-chymotrypsin (CIN-CT). The difference spectrum between CIN-CT and α-chymotrypsin at pH 2.0, 21°C, gives a peak with a maximum at 290 nm. At 51°C, the same difference spectrum peaks at 281 nm, i.e., at the same positions as that of the model com-

128. D. B. Wetlaufer, J. T. Edsall, and B. R. Hollingworth, *JBC* **233**, 1421 (1958).

129. M. Burr and D. E. Koshland, Jr., *Proc. Natl. Acad. Sci. U. S.* **52**, 1017 (1964).

130. J. Mercouroff and G. P. Hess, *BBRC* **11**, 283 (1963).

pound *O*-CIN-*N*-acetyl serineamide, in which the cinnamoyl group is exposed to solvent. This spectral shift suggests that in the enzyme–substrate compound the CIN group is not in contact with solvent at room temperature, becoming exposed only after thermal unfolding. This conclusion was confirmed by solvent perturbation spectroscopy experiments. When 20% ethylene glycol or 20% glycerol were used as perturbants, the solvent perturbation difference spectrum of native CIN-CT was identical with that of native α-chymotrypsin; thus, the cinnamoyl group was not coming in contact with solvent. Disruption of the tertiary structure of the CIN–enzyme compound by peptic hydrolysis was accompanied by the generation of a solvent perturbation difference spectrum, i.e., by the exposure to solvent of the bound substrate. These observations indicate that binding of the cinnamoyl group to the active site of the enzyme results in its complete burial within the native enzyme. Had it been present either in a crevice or in a partly exposed state, the small perturbants used would have generated a typical cinnamoyl difference spectrum. The removal from contact with solvent of the substrate chromophoric group when it is bound to the enzyme is consistent with the occurrence of a local conformational change. Such a structural change had been detected previously by Havsteen and co-workers (*131*, *132*) who found by optical rotatory dispersion and difference spectroscopy large differences between the thermodynamic parameters of structural transitions of the enzyme and enzyme–substrate compounds, when they probed the systems α-chymotrypsin, monoacetyl-α-chymotrypsin, and diisopropylphosphoryl-α-chymotrypsin.

Comparison of the results obtained with two probing techniques (circular dichroism and difference spectroscopy) permits more detailed conclusions to be drawn on the state of particular amino acids within the enzyme. When chymotrypsinogen is activated to chymotrypsin or when the enzyme reacts with substrates, the environments of both tyrosine and tryptophan residues change. Yet, the activation process does not affect the near ultraviolet tryptophan circular dichroism spectrum (*71*, *72*) (see Fig. 7). Chymotrypsinogen has eight tryptophans. *N*-Bromosuccinimide oxidation (*110*) permits their grouping into two classes: three residues are fully exposed and five are essentially buried. The optical activity must therefore reside fully in the buried tryptophans. These must be more constrained in space than the exposed ones, whose freedom of motion may be sufficient to mutually cancel CD absorption bands (*70*).

131. B. H. Havsteen and G. P. Hess, *JACS* **85**, 791 (1963).
132. B. Havsteen, B. Labouesse, and G. P. Hess, *JACS* **85**, 796 (1963).

VII. Fluorescence Spectroscopy

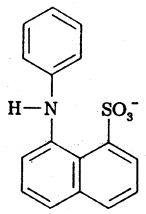
One of the most useful and versatile probes of enzyme conformation in solution is fluorescence spectroscopy. Since its application by Weber (*133–138*) to protein studies, this technique has undergone extensive development and has found wide uses in the probing of various structural problems which arise in enzyme chemistry. The scope and applicability of this technique has been discussed in a number of reviews (*133, 136, 139–143a*). Briefly, the types of structural information which may be obtained include the extent of flexibility of a protein and the hydrodynamic volume of its subunits; the degree of polarity of particular regions, e.g., the active site of an enzyme; the distances between specific groups on a protein as well as complexed ligands and specific interactions which occur between them; the quarternary structure of a protein and the rate of very rapid conformational transitions. The various kinds of structural information are obtained from determinations of the excitation and emission spectra of fluorescent groups attached to proteins, in particular, from the band positions and intensities under various circumstances, the transfer of energy from one emitter to another, the effect of the protein environment on quantum yield, including quenching of fluorescence by vicinal groups such as ionizable amino acid residues, and the rate of decay of the parallel and perpendicular components of fluorescent radiation.

The fluorescent chromophores which are used in enzyme studies are of three types: intrinsic, i.e., the tryptophan, tyrosine and phenylalanine

133. G. Weber, *in* "Light and Life" (W. D. McElroy and B. Glass, eds.), p. 82. Johns Hopkins Press, Baltimore, Maryland, 1961.
134. G. Weber, *BJ* **51,** 145 (1952).
135. G. Weber, *BJ* **51,** 155 (1952).
136. G. Weber, *Advan. Protein. Chem.* **8,** 415 (1953).
137. G. Weber and D. J. R. Lawrence, *BJ* **56,** 31P (1954).
138. G. Weber and L. B. Young, *JBC* **239,** 1415 (1964).
139. S. V. Konev, "Fluorescence and Phosphorescence of Proteins and Nucleic Acids." Plenum Press, New York, 1967.
140. S. Udenfriend, "Fluorecence Assay in Biology and Medicine." Academic Press, New York, 1965.
141. L. Brand, "Methods in Enzymology," Vol. 11, p. 776, 1967.
142. L. Stryer, *Science* **162,** 526 (1968).
143. R. F. Chen, *in* "Fluorescence" (G. G. Guilbault, ed.), p. 443. Marcel Dekker, New York, 1967.
143a. R. F. Chen, H. Edelhoch, and R. F. Steiner, *in* "Physical Principles and Techniques of Protein Chemistry" (S. J. Leach, ed.), Part A, p. 171. Academic Press, New York, 1969.

residues of the amino acid sequence; cofactors such as some coenzymes; and, extrinsic chromophores. The last type includes a number of compounds which are artificially incorporated (either by covalent or by noncovalent bonds) into specific binding sites on the enzyme. While the first two types of chromophoric groups are very useful as probes of structural features (*133*, *139*, *141*), they are not always found in the three-dimensional structure in locations or orientations which are favorable for the scrutiny of particular structural details. To overcome this problem, Weber (*135*) introduced the technique of binding polycyclic aromatic compounds to the protein in question and using their fluorescence properties as structural probes. If such a group is part of an enzyme, substrate or inhibitor, it can be incorporated specifically into the active site and used to great advantage to obtain information on the local structure and interactions within the site.

The determination of the polarity of a locus on an enzyme is based on the knowledge that a change in polarity of the immediate environment of the emitting group results in a displacement of the position of the fluorescence emission spectral band. For example, Stryer (*144*) has shown that the position of the emission band of 1-anilino-8-naphthalene sulfonate



(II)

(ANS) was shifted to longer wavelengths as the polarity of solvent increased from that of *n*-octanol ($\lambda_{max} = 464$ nm) to that of ethylene glycol ($\lambda_{max} = 484$ nm). This red shift in band position is accompanied by a decrease in the quantum yield. The proximity of groups within an enzyme is measured by the efficiency of energy transfer between them. It is found that when the transfer is from the singlet state of one group to the singlet state of another group, the distances that may be measured are between 10 and 65 Å. Förster (*145*) treated this case quantitatively. Regarding singlet–singlet transfer as occurring via the interaction of dipoles on the donor and acceptor, he defined a distance R_0 (at which

144. L. Stryer, *JMB* **13**, 482 (1965).
145. T. Förster, *Ann. Physik*, [6]**2**, 55 (1948).

transfer is 50% efficient) in terms of spectroscopic and geometric parameters. Thus,

$$R_0 = 9.79 \times 10^3 (J n^{-4} \kappa^2 Q)^{1/6} \quad (1)$$

where J is the integral of overlap of the emission spectrum of the donor group and the absorption spectrum of the acceptor group, n is the refractive index of the medium, κ^2 is a factor defined by the mutual orientation of the two dipoles (it may vary between 0 and 4; for random orientation, its value is 2/3), and Q is the quantum yield of the donor. If the observed efficiency of transfer between two groups is e, then the distance between them, r, is $r = (e^{-1} - 1)^{1/6} R_0$. Recently, these relations have been examined critically (*146*, *147*). In a very elegant study, Stryer and Haugland (*148*) tested Förster's theory on a system which consisted of a donor (α-naphthyl) and an acceptor (dansyl) group coupled to two ends of polyproline chains of known different lengths and were able to confirm fully the r^{-6} dependence. Systems in which the energy transfer is triplet–singlet, act over similar distances (*149*). In the case of triplet–triplet energy transfer, the effective distance is much shorter, less than about 12 Å, since the mechanism is by an electron–exchange interaction. Thus, with the proper donor and acceptor groups, fluorescence spectroscopy provides a set of rulers for measuring specific distances on enzyme molecules (*142*).

Information on the rigidity of structure, molecular shape, and presence of mobile structural segments of an enzyme molecule results from measurements of the decay of fluorescence polarization (*134–136*, *150*, *151*). The enzyme solution is illuminated with polarized radiation, exciting those molecules in which the absorbing groups are oriented preferentially in a direction parallel to the plane of polarization of the light, and the intensities of fluorescence parallel and perpendicular to the incident light are measured as a function of time. This gives the anisotropy parameter $A(t)$ at any moment t:

$$A(t) = \frac{I_\parallel(t) + 2I_\perp(t)}{I_\parallel(t) - I_\perp(t)} \quad (2)$$

where $I_\parallel(t)$ and $I_\perp(t)$ are the components of fluorescence parallel and perpendicular to the plane of polarization of the incident energy at any

146. J. Eisinger, *Biochemistry* **8**, 3902 (1969).
147. J. Eisinger, B. Feuer, and A. A. Lamola, *Biochemistry* **8**, 3908 (1969).
148. L. Stryer and R. P. Haugland, *Proc. Natl. Acad. Sci. U. S.* **58**, 719 (1967).
149. R. G. Bennett, R. P. Schwenker, and R. E. Kellogg, *J. Chem. Phys.* **41**, 3040 (1964).
150. A. Jablonski, *Z. Naturforsch.* **16a**, 1 (1961).
151. R. P. Haugland and L. Stryer, *in* "Conformation of Biopolymers" (G. N. Ramachandran, ed.), Vol. 1, p. 321. Academic Press, New York, 1967.

time t. This is related to the relaxation times ρ_i of various mobile components of the molecule by

$$A(t) = A_0 \sum_i \alpha_i \exp(-3t/\rho_i) \tag{3}$$

where A_0 is the anisotropy at time zero, t is the time, and α_i is the fractional contribution of relaxation i. For a spherical molecule, the relaxation time ρ_0 is related to molecular volume V since $\rho_0 = 3\eta V/kT$, where η is the solution viscosity, k is Boltzmann's constant, and T is the thermodynamic temperature. If the exciting radiation consists of an almost instantaneous flash (of duration of the order of a few nanoseconds), the anisotropy $A(t)$ can be measured directly since the duration of the fluorescence decay is usually of the order of 10–100 nanoseconds (nsec, 10^{-9} sec) (*142, 151–156*).

Not only is fluorescence a highly versatile technique, capable of yielding a wealth of structural information, but also it has the further advantages that measurements can be made at very low protein concentration. Difference spectroscopy normally requires working at total optical densities of the order of two; in the case of fluorescence, it is possible to work at enzyme concentrations an order of magnitude lower. Furthermore, the fluorescence spectrum reveals only the state of the excited molecules so that one sees the net effect of interaction of the molecules with the light. There is no need for any corrections resulting from overlap with incident radiation nor with spectra of molecules in the ground state. Let us examine now with the aid of specific examples the type of structural information that may be obtained.

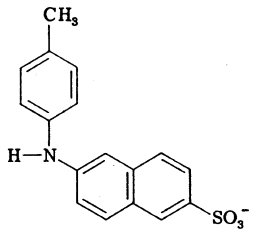
A. Typical Cases

1. *Chymotrypsin*

The structure of chymotrypsin has been probed by McClure and Edelman (*157–159*) and Stryer and co-workers (*142, 151, 160, 161*). McClure

152. L. Hundley, T. Coburn, E. Garwin, and L. Stryer, *Rev. Sci. Instr.* **38**, 488 (1967).
153. H. Lami, G. Pfeffer, and G. Laustriat, *J. Phys.* (*Paris*) **27**, 398 (1966).
154. P. Wahl, *BBA* **175**, 55 (1969).
155. P. Wahl, *Compt. Rend.* **260**, 6891 (1965); **263**, 1525 (1966).
156. P. Wahl and S. N. Timasheff, *Biochemistry* **8**, 2945 (1969).
157. W. O. McClure and G. M. Edelman, *Biochemistry* **5**, 1908 (1966).
158. W. O. McClure and G. M. Edelman, *Biochemistry* **6**, 559 (1967).
159. W. O. McClure and G. M. Edelman, *Biochemistry* **6**, 567 (1967).

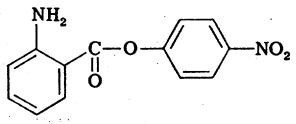
and Edelman examined the activation of chymotrypsinogen to chymotrypsin using as probe 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS)



(III)

which is a specific fluorescent marker of hydrophobic regions (*157*). When solutions of chymotrypsinogen containing TNS were activated by the addition of trypsin ("fast" activation leading to δ-chymotrypsin), the fluorescence intensity increased 20-fold at a rate parallel with the appearance of chymotrypsin activity (*159*). It is apparent that on activation a structural change occurs, in which the proper hydrophobic environment for TNS is generated. Fluorescence of TNS-chymotrypsin was inhibited by the binding of substrate analogs, while binding of TNS to the enzyme inhibited noncompetitively the hydrolysis of acetyl-L-tyrosine ethyl ester, suggesting the presence of a hydrophobic binding site which is not part of the active site of the enzyme (*158*). When the pH was varied between 7 and 9, the fluorescence intensity decreased sharply, suggesting that the ionizing groups which influence fluorescence may be the same as those which control enzyme activity (*158*). It is interesting to recall that circular dichroism studies reveal a conformational change related to the activation of the enzyme, as well as to the state of ionization of key groups such as Ile 16 and Asp 194 (*72, 77, 79, 80*).

Haugland and Stryer (*151*) probed the polarity and flexibility of the active site of α-chymotrypsin. By reacting *p*-nitrophenyl anthranilate



(IV)

with the enzyme, they formed a fluorescent anthraniloyl derivative, in which the probing group was attached to the active site at Ser 195, and which was highly stable at neutral pH. The anthraniloyl group is particularly favorable for this kind of study. Its absorption and emission maxima at 342 and 422 nm are remote from those of the protein aromatic residues, which makes it possible to excite the probing group exclusively. By applying a nanosecond pulse (*142*, *151*), the decay curves of the parallel and perpendicular components of the fluorescence were established, resulting in the dependence of the anisotropy $A(t)$ on time, as shown in Fig. 17. When the shape and finite duration of the exciting flash

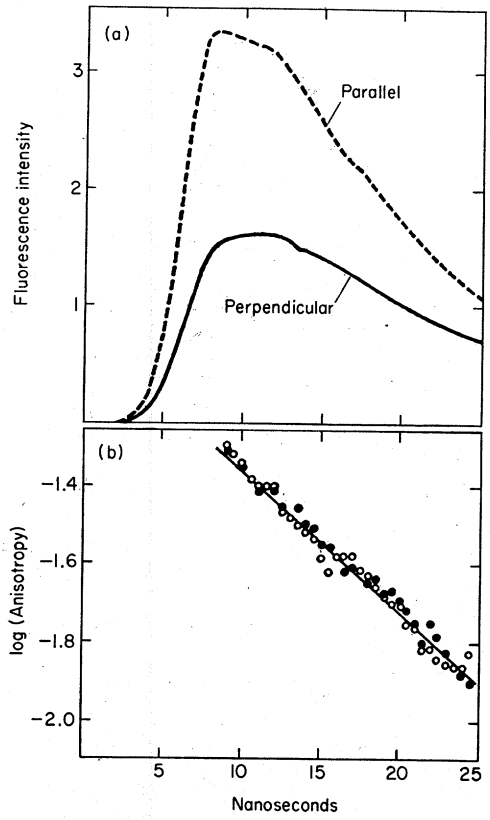


FIG. 17. Nanosecond fluorescence polarization of anthraniloyl chymotrypsin in 0.1 *M* phosphate buffer, pH 6.8, at 22°C. (a) Intensity of the parallel and perpendicularly polarized components of the fluorescence as a function of time. (b) Logarithm of the emission anisotropy as a function of time. The slope (when corrected for the finite duration of the light pulse) yields a rotational relaxation time of 52 nsec for the anthraniloyl chromophore [from Stryer (*142*)].

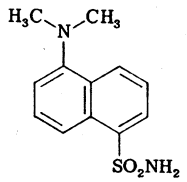
were accounted for by means of a convolution analysis (*152*, *154*, *156*), a rotational relaxation time of 52 nsec was obtained for the anthraniloyl group when it is attached to the enzyme. This value of ρ permitted the immediate elimination of molecular models in which the active site region of the enzyme is highly flexible. Indeed the expected value of ρ_0 at 20°C for an anhydrous spherical protein molecule with a molecular weight of 25,000 daltons is 22 nsec. Since the experimental value is more than twice that calculated for a rigid sphere, it must be concluded that the anthraniloyl chromophore is rigidly fixed to the active site of the enzyme and has only that rotational mobility which is part of the mobility of the whole enzyme. Furthermore, the high value of the relaxation time indicates that α-chymotrypsin is a hydrated nonspherical molecule, which is perfectly consistent with the known three-dimensional structure (*16*). The emission maximum of the anthraniloyl derivative in 1.0 *M* phosphate buffer of pH 6.8 is at 422 nm, i.e., very close to the position of methyl anthranilate in water (420 nm). Since the enclosure of this group in nonpolar environment results in a blue shift of its emission maximum (for methyl anthranilate the positions are 405 nm in methanol, 390 nm in dioxane, and 380 nm in cyclohexane), Haugland and Stryer (*151*) concluded that the environment of this group, when it is coupled to α-chymotrypsin, is highly polar; furthermore, the observed shifts of the absorption maximum indicate that water is excluded from the binding site. These conclusions are entirely reasonable since the structural region of α-chymotrypsin in the vicinity of the active site contains a number of polar, or even ionized, residues (e.g., Ile 16, Asp 194, Ser 195, and His 57), and it is possible that the anthraniloyl group is located in their vicinity rather than in a hydrophobic part of the cavity.

The same fluorescent probe was used to measure the distance between tryptophan residues and the active site. The excitation spectrum of the anthraniloyl derivative overlaps the emission spectrum of tryptophan residues. By applying the Förster theory (*145*) to this singlet–singlet energy transfer, Haugland and Stryer found a characteristic distance R_0 of 20 Å which, however, cannot be decomposed into distances between individual tryptophans and the active site. A further elaboration of this problem was provided by Galley and Stryer (*161*) who probed this distance by triplet–triplet energy transfer from *m*-acetylbenzenesulfonamide, covalently bonded to Ser 195 of α-chymotrypsin, to tryptophan residues of the enzyme. This system is amenable to such studies because the triplet level of the sulfonamide is higher than that of tryptophan, while the opposite is true of the singlet levels. Excitation of the probing chromophore resulted in no tryptophan emission, showing that none of the tryptophans are in the vicinity of the active site of the enzyme, a conclusion in agreement with the X-ray diffraction results (*16*).

2. *Carbonic Anhydrase*

Another interesting example of the fluorescence probing of an enzyme active site is found in the studies on bovine carbonic anhydrase. Using the same triplet–triplet energy transfer system as with α-chymotrypsin, Galley and Stryer (*161*) attached to the active site *m*-acetylbenzenesulfonamide, a known specific inhibitor. Excitation of the marker at 330 nm, where tryptophan does not absorb, resulted only in tryptophan phosphorescence with an energy transfer efficiency of close to 100%. This means that a tryptophan residue is present at the active site of carbonic anhydrase, or close to it.

In a similar study, Chen and Kernohan (*104*) probed the active site



(V)

of bovine carbonic anhydrase B with the inhibitor, 5-dimethylaminonaphthalene-1-sulfonamide (DNSA). In water DNSA has an emission maximum at 580 nm and a quantum yield of 0.055. Incorporation into the enzyme resulted in a blue shift to 468 nm and a dramatic increase of the quantum yield to 0.84. Both changes show that contrary to the case of α-chymotrypsin the binding site is highly nonpolar, while the high quantum yield indicates in addition that the site is shielded from solvent interactions. The efficiency of energy transfer from the seven tryptophans to the single DNSA group of 85% suggests that all the tryptophans are within the critical transfer distance R_0 (*145*) from the probe and probably in favorable mutual orientation. The observed quenching of 73% of tryptophan fluorescence by the DNSA led Chen and Kernohan to conclude that the fluorescence efficiencies of the seven tryptophans are different and that the relative coordinates of the bound DNSA and the tryptophans are such that energy transfer is more probable from the less fluorescent residues. Measurements of fluorescence decay times gave a relaxation time of 30 nsec; therefore, the inhibitor is rigidly attached to the enzyme, which has a symmetrical hydrodynamic structure. These conclusions on the nature of the inhibitor binding site are fully in accord with those drawn from circular dichroism experiments (*46*) and with the known features of the three-dimensional structure deduced from X-ray crystallographic analysis (*102*), which shows that the inhibitor lies within

a deep crevice in the enzyme with the sulfonamide group bound to the Zn ion. This has led Chen and Kernohan (*104*) to conclude with the observation that ". . . if the active site and the sulfonamide-binding site are indeed identical, we are left with the mild paradox that water reacts at or near a hydrophobic site." That this "paradox" may be true is further substantiated in the literature (*103*).

3. *Lysozyme*

Details of the interaction of lysozyme with substrate molecules have been examined by Lehrer and Fasman (*162*, *163*) who used direct and differential fluorescence spectroscopy methods (*163a*). It is known that three of the six tryptophans of this enzyme are located in the region of the active site (*164*, *165*). Binding of substrates or inhibitors results in alterations of the circular dichroism spectrum (*166*), a red shift in the ultraviolet absorption spectrum (*167*, *168*) and changes in tryptophan fluorescence (*162*, *163*, *169–171*). Lehrer and Fasman (*162*, *163*) examined the tryptophan fluorescence of lysozyme as a function of pH in the presence of ligands of increasing size. In the native enzyme, the quantum yield is very low, indicating considerable quenching. As the size of the ligand is increased, the intensities of the emission spectra are progressively enhanced and their maxima are shifted to lower wavelengths. The fluorescence intensities of this enzyme, as well as of its complexes with di-*N*-acetyl-D-glucosamine (diNAG) and tri-*N*-acetyl-D-glucosamine (triNAG), are shown in Fig. 18 as a function of pH. It is evident that binding of substrate greatly increases the fluorescence at neutral pH. A decrease in pH leads to a two-step quenching, between pH 7 and 5.5 and between pH 4 and 2, while an increase in pH above 8 also causes a sharp drop in fluorescence intensity. Analysis of difference spectra between lysozyme-triNAG complexes at various pH values (shown in Fig. 19) per-

162. S. S. Lehrer and G. D. Fasman, *BBRC* **23**, 133 (1966).
163. S. S. Lehrer and G. D. Fasman, *JBC* **242**, 4644 (1967).
163a. B. Bablouzian, M. Grourke, and G. D. Fasman, *JBC* **245**, 2081 (1970).
164. L. N. Johnson and D. C. Phillips, *Nature* **206**, 761 (1965).
165. D. C. Phillips, *Sci. Am.* **215**, 78 (1966).
166. A. N. Glazer and N. S. Simmons, *JACS* **88**, 2335 (1966).
167. K. T. Hayashi, G. Imoto, and M. Funatsu, *J. Biochem.* (*Tokyo*) **54**, 381 (1963); **55**, 516 (1964).
168. F. W. Dahlquist, L. Jao, and M. A. Raftery, *Proc. Natl. Acad. Sci. U. S.* **56**, 26 (1966).
169. R. F. Steiner and H. Edelhoch, *Nature* **192**, 873 (1961).
170. M. Shinitzky, V. Grisaro, D. M. Chipman, and N. Sharon, *ABB* **115**, 232 (1966).
171. R. F. Steiner and H. Edelhoch, *BBA* **66**, 341 (1963).

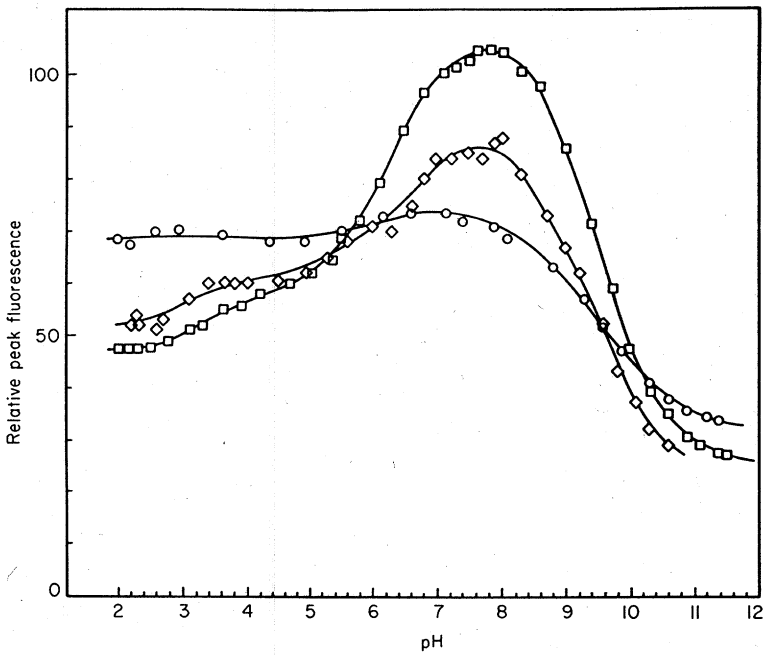


FIG. 18. Fluorescence plotted against pH: (○) lysozyme; (◇) lysozyme + 0.1% di-*N*-acetyl-D-glucosamine; and (□) lysozyme + 0.1% tri-*N*-acetyl-D-glucosamine. Enzyme emission at 345 nm; enzyme + di-*N*-acetyl-D-glucosamine emission at 337 nm; enzyme + tri-*N*-acetyl-D-glucosamine emission at 335 nm. In 0.2 *M* NaCl at 25°. Lysozyme concentration, 0.005%, $OD_{280} \approx 0.10$. Excitation at 280 nm, 1 cm path length cell. Relative fluorescence for same concentration of enzyme [from Lehrer and Fasman (*163*)].

mitted the conclusion that in complex with trimer two tryptophan residues are quenched independently by two abnormal carboxylic groups, one with an unusually high pK (~6.3) and the other with a low pK (~3.0) (*172*, *173*); the high pH quenching is most probably due to energy transfer to a tyrosine residue (*174*) with a pK of 9.95. It is known that protonated carboxyls can quench tryptophan fluorescence (*175*, *176*). The conclusion on the interaction of two different tryptophans with two carboxyls is based on the formation of different difference spectra for the two pH intervals. The difference spectra were analyzed in the following way. The

172. J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *JACS* **82**, 2154 (1960); **83**, 2686 (1961).
173. C. Tanford, *Advan. Protein Chem.* **17**, 69 (1962).
174. R. Cowgill, *BBA* **94**, 81 (1965).
175. G. D. Fasman, E. Bodenheimer, and A. Pesce, *JBC* **241**, 916 (1966).
176. A. White, *BJ* **71**, 214 (1959).

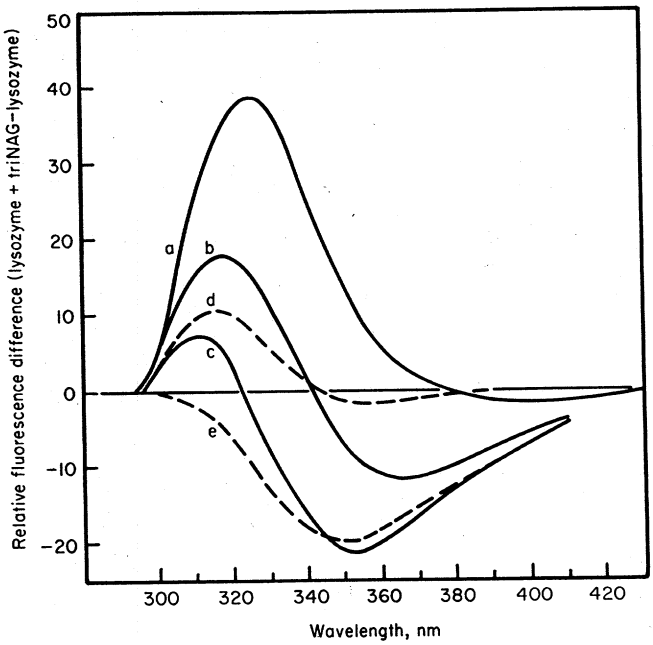


FIG. 19. Fluorescence difference spectra at different pH values caused by binding tri-*N*-acetyl-D-glucosamine (triNAG) (F[lysozyme + tri-*N*-acetyl-D-glucosamine] − F[lysozyme]). Curves a, pH 7.5; b, pH 5.5; c, pH 2.0; and d and e decomposition of c into two contributions [from Lehrer and Fasman (*163*)].

negative contribution to the pH 2.0 difference spectrum (curve e, Fig. 19) is the fluorescence of two residues in the free enzyme which are quenched on formation of the complex; the positive contribution (curve d, Fig. 19) corresponds to the spectral shift of the third residue. The peak of the quenched fluorescence (curve e) is at 345 nm, indicating presence of the corresponding tryptophan in an aqueous environment when it is not complexed with the substrate. Furthermore, the tryptophan residues which are preferentially quenched by ionized tyrosines are located in a nonaqueous environment since the energy transfer occurs in the wavelength region below 320 nm, which is characteristic of the fluorescence of nonpolar-type tryptophans. Comparison of the difference fluorescence data (*163*) with X-ray structural information (*165*) has led Lehrer and Fasman to postulate that the interactions observed in the quenching experiments are between Trp 108 and Glu 35 (high pK) and Trp 63 and Asp 101 (low pK), although interaction between Trp 63 and Asp 52 is also possible. Imoto and Rupley (*177*) have shown, by a combination of

177. T. Omoto and J. A. Rupley, *Federation Proc.* 27, 392 (1968).

difference spectroscopy with chemical modification experiments, that the difference spectrum of lysozyme near 300 nm is a function of the interaction between Trp 108 and Glu 35. That the difference spectra of tryptophan-containing proteins are perturbed near 300 nm by the interaction of charges with tryptophan residues has been demonstrated recently by Ananthanarayan and Bigelow (*178, 179*).

4. *Staphylococcal Nuclease*

An interesting study of active-site probing by various physical techniques has been carried out by Anfinsen and co-workers on the nuclease from *Staphylococcus aureus* (*126, 180*). Here we shall limit our discussion to the fluorescence analysis (*180, 181*). It was found that addition of the inhibitor, deoxythymidine 3′,5′-diphosphate (pdTp) greatly suppresses the tyrosine fluorescence without affecting the region of tryptophan emission (*171*). Following their solvent perturbation spectroscopy observation that binding of the nucelotide leads to the burial of all but two tyrosine residues (*126*), the authors carried out solvent perturbation experiments using fluorescence as the criterion of exposure. The fluorescence of the model compound, acetyltyrosinamide, is strongly enhanced by solvents of low polarity (*180*). When the fluorescence intensity of the enzyme and of the enzyme–inhibitor complex was measured in similar fashion as a function of ethanol concentration, the change observed with the free enzyme was almost identical to that found with the model compound, while for the complex the effect was much smaller, confirming the conclusion that complexing buries some tyrosine residues. Again, there was no effect on the single buried tryptophan. Binding of pdTp also resulted in a decrease of the relaxation time from 17.7 to 15.6 nsec, pointing to a small conformational change which increases the hydrodynamic symmetry of the enzyme molecule.

B. Conclusion

As is evident from the specific examples cited, fluorescence is a powerful probe of enzyme structure and enzyme–ligand interactions. This method can be used as a ruler to measure distances between specific

178. V. S. Ananthanarayan and C. C. Bigelow, *Biochemistry* **8**, 3717 (1969).
179. V. S. Ananthanarayan and C. C. Bigelow, *Biochemistry* **8**, 3723 (1969).
180. P. Cuatrecasas, H. Taniuchi, and C. B. Anfinsen, *Brookhaven Symp. Biol.* **21**, 172 (1969).
181. P. Cuatrecasas, H. Edelhoch, and C. B. Anfinsen, *Proc. Natl. Acad. Sci. U. S.* **58**, 2043 (1967).

groups as was done with α-chymotrypsin and carbonic anhydrase: It can detect specific interactions between amino acid residues, as in lysozyme, and it can measure molecular dimensions and rigidity and probe for the exposure to solvent of aromatic residues on an enzyme. Other possible applications are to the specific detection of given secondary structures (*182*), to the quarternary structures of subunit systems (*156*), and to the analysis of substrate and subunit interactions (*183*). As is true of all other solution physical techniques, however, fluorescence in all its ramifications becomes a most powerful probe when used in conjunction with other methods.

VIII. Ionizable Groups

The environment of ionizable groups of enzymes and changes in their state is probed most easily by measurements of their interactions with protons as the concentration of the latter is varied, i.e., by a determination of their titration curves. General acid–base titration curves have been widely used to characterize enzymes, and their detailed discussion is available in a number of reviews (*173*, *184–189*); therefore, this presentation will be limited to the examination of some specific groups, with particular emphasis on the identification of ionizable residues involved in interactions.

While the determination of the proton binding capacity of an enzyme as a function of pH gives information on the overall electrostatic state of the molecule and its changes resulting from structural transitions, the ionization of particular groups and their structural environment may be examined quite readily with the help of spectroscopic differences between

182. J. Lynn and G. D. Fasman, *BBRC* 33, 327 (1968).
183. M. E. Goldberg, S. York, and L. Stryer, *Biochemistry* 7, 3662 (1968).
184. E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Chapter 20, p. 444. Reinhold, New York, 1943.
185. J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. 1, Chapters 8 and 9. Academic Press, New York, 1958.
186. C. Tanford, "Physical Chemistry of Macromolecules," Chapters 7 and 8. Wiley, New York, 1961.
187. S. A. Rice and M. Nagasawa, "Polyelectrolyte Solutions." Academic Press, New York, 1961.
188. J. Steinhardt and S. Beychok, *in* "The Proteins" (H. Neurath, ed.), 2nd ed., Vol. 2, Chapter 8. Academic Press, New York, 1964.
189. S. N. Timasheff, *in* "Biological Polyelectrolytes" (A. Veis, ed.), p. 1. Marcel Dekker, New York, 1970.

their ionized and unionized states. The titration curves of tyrosine (*190*), histidine (*191*), and cysteine (*192*) residues may be obtained by ultraviolet difference spectroscopy, ionization of carboxylic groups can be followed in the infrared region (*193*), and histidine imidazoles have been titrated as well with nuclear magnetic resonance (NMR) as detector (*194–196*). Nonchromophoric ionizable residues may be titrated spectroscopically if their state of ionization perturbs the absorption or emission behavior of chromophores with which they interact (*197*); a typical example of such a titration has been described above (see Section VII,A,3) in the case of two abnormal carboxyls in lysozyme (one with a high pK, the other with a low pK) which perturb the fluorescence of specific tryptophan residues (*162*, *163*).

The titration of chromophoric residues is rendered possible by the displacement of the absorption maxima to higher wavelengths when the pertinent groups become electrostatically charged. The positions of the bands are listed in Table V. Further details may be found in the review literature (*8*, *107*, *109a*, *198*). The normal procedure consists in the determination of absorption spectra of the enzyme measured at various pH values using as reference an enzyme solution of identical concentration at a pH at which the groups in question are either all ionized or

TABLE V
ABSORPTION MAXIMA OF IONIZING CHROMOPHORES

Residue	Chromophore	Uncharged (nm)	Charged (nm)
Tyrosine	Phenolic	193	200
		222	235
		274	295
Histidine	Imidazole	211	237
Cysteine	Sulfhydryl	195	235
Asp, Glu	Carboxyl	1710 cm^{-1} [a]	1560 cm^{-1} [a]

[a] Infrared.

190. J. L. Crammer and A. Neuberger, *BJ* **37**, 302 (1943).
191. J. W. Donovan, *Biochemistry* **4**, 823 (1965).
192. J. W. Donovan, *Biochemistry* **3**, 67 (1964).
193. H. Susi, T. Zell, and S. N. Timasheff, *ABB* **85**, 437 (1959).
194. J. H. Bradbury and H. A. Scheraga, *JACS* **88**, 4240 (1966).
195. D. H. Meadows, J. L. Markley, J. S. Cohen, and O. Jardetzky, *Proc. Natl. Acad. Sci. U. S.* **58**, 1307 (1967).
196. D. H. Meadows, O. Jardetzky, R. M. Epand, H. H. Ruterjans, and H. A. Scheraga, *Proc. Natl. Acad. Sci. U. S.* **60**, 766 (1968).
197. J. W. Donovan, *Biochemistry* **6**, 3918 (1967).
198. G. H. Beaven and E. R. Holiday, *Advan. Protein Chem.* **7**, 319 (1952).

all unionized. The difference in absorption $\Delta\epsilon(\lambda)$ at a given wavelength λ is then directly related to the degree of ionization α since

$$\Delta\epsilon(\lambda) = [\epsilon_i(\lambda) - \epsilon_u(\lambda)]\alpha \quad (4)$$

where $\epsilon_i(\lambda)$ and $\epsilon_u(\lambda)$ are the molar absorption coefficients of the group in question in the ionized and unionized states, respectively.

Spectrophotometric titrations have been used extensively in the identification of normally and abnormally ionizing residues. Normal groups are usually exposed to solvent; they ionize with the intrinsic pK values obtained with the same residues when included in small peptides. Abnormally titrating groups are those which, because of interactions, ionize with apparent pK values which are either too high or too low; their ionization properties are normalized only by conformational changes. The interactions may be of various natures. Ionizable groups may be buried within the nonpolar folds of the protein molecule; such groups must remain neutral, even though the pH of the surrounding solvent corresponds to their ionized state, since the burial of a charge within the interior of a protein introduces a structure destabilizing free energy contribution of 50–100 kcal/mole (*84*), i.e., more than the net free energy of stabilization of a globular protein. If a group is buried in such manner, its pK is raised if it is anionic; the pK is lowered if the group is cationic. Typical examples are the abnormal tyrosines found in many enzymes, e.g., in bovine and human carbonic anhydrase (*24, 199*), as well as the buried histidines of the same enzyme (*199*). A pair of charged groups of opposite sign may be buried as an ion pair; in this case, the pK of the cationic group is raised and that of the anionic group is lowered. A typical example is found in the ion pair formed between Ile 16 and Asp 195 of chymotrypsin and DIP-chymotrypsin (*80, 85*). Ionizable groups may also interact via the formation of hydrogen bonds; the variation of the pK values in this case depends on whether the group is hydrogen bonded in the charged or neutral state and on whether it is the donor or acceptor; a typical example is found in the tyrosine–carboxyl interactions of ribonuclease (*200*). Furthermore, the pK of a group may be shifted significantly if it is located in the immediate vicinity of another charged group while fully in contact with solvent.

Typical titration spectra are shown in Fig. 20 for the ionization of pepsinogen tyrosines (*201*). In Fig. 20a the direct spectra of the zymogen

199. L. M. Riddiford, R. H. Stellwagen, S. Mehta, and J. T. Edsall, *JBC* **240,** 3305 (1965).

200. J. P. Riehm, C. A. Broomfield, and H. A. Scheraga, *Biochemistry* **4,** 760 (1965).

201. G. E. Perlmann, *JBC* **239,** 3762 (1964).

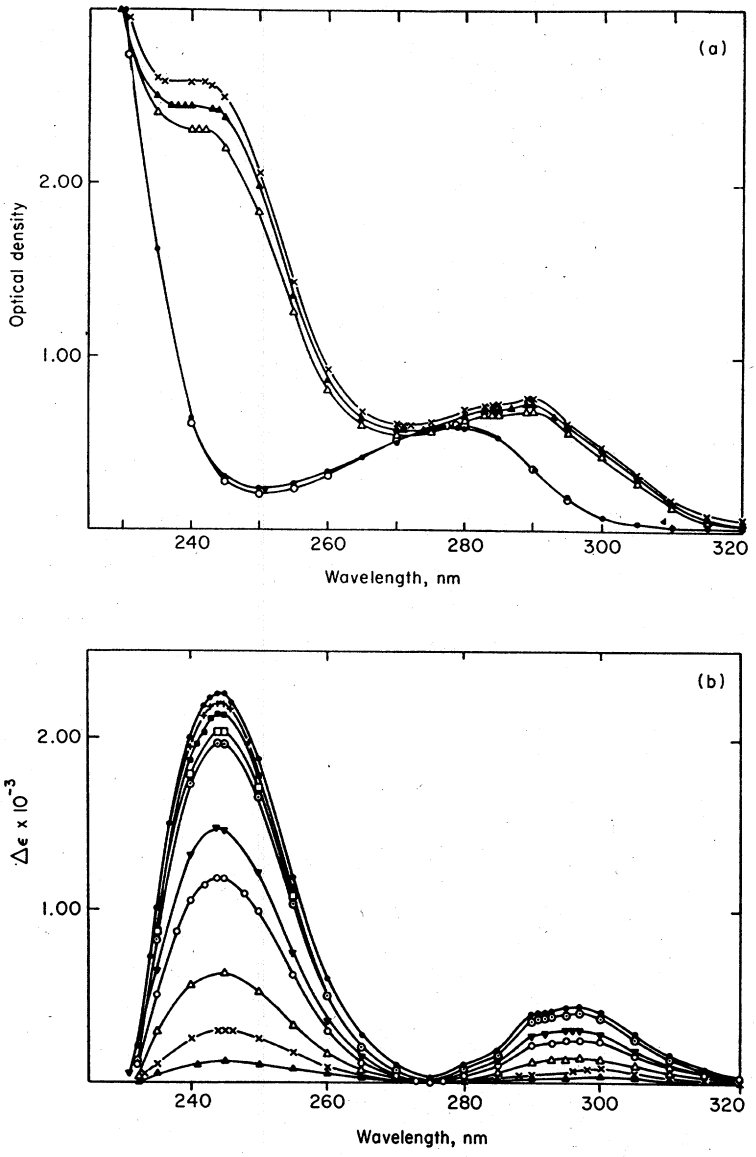


FIG. 20. (a) Dependence of the ultraviolet spectra of pepsinogen on pH (pH values as follows): (○) 8.36, (●) 9.25, (△) 11.75, (▲) 12.12, and (×) 12.56. (b) Dependence of the ultraviolet difference spectra of pepsinogen on pH (pH values as follows): (▲) 9.62, (×) 10.03, (△) 10.29, (○) 10.85, (▼) 11.29, (⊙) 11.75, (□) 11.93, (■) 12.12, (+) 12.38, and (●) 12.60. Both (a) and (b) from Perlmann (*201*).

show that as pH is raised the UV spectrum displays a general red shift and enhancement of absorption above 230 nm: new absorption maxima appear near 243 and 292 nm. The difference between these spectra and that of a standard pepsinogen solution at pH 7.7 is shown in Fig. 20b: Difference bands of increasing intensity with increasing pH are observed at 245 and 295 nm. Application of Eq. (4) to these data results in the titration curve of pepsinogen tyrosines. Comparison of the titration curves of this enzyme precursor obtained in dilute buffers and in the presence of urea revealed that while 15 tyrosine residues are normally available to solvent, two are embedded in the interior of the molecule. This conclusion is consistent both with the analysis of chemical modification studies (*202–204*), which permit a gradation in the state of exposure of these groups, and with observations on conformational transitions which occur in the alkaline range (*205–207*).

In making differential spectroscopic measurements, it is very important to choose for reference a solution in the proper state (*192*, *208*). When abnormal groups are titrated, their spectra change because of (1) the ionization of the group and (2) transfer of the group from a state of interaction either with other groups or with the nonpolar interior of the protein to contact with solvent; the second effect frequently results in a perturbation difference spectrum without a change in the state of ionization. The change in pH may also result in the perturbation of the absorption spectra of other chromophores which absorb in the wavelength region of interest; these spectral changes evidently also contribute to the observed overall difference spectrum, and if not properly corrected for they lead to erroneous conclusions on the ionization of the groups of interest. Typical examples are found in the perturbation of tryptophan absorption when the tyrosines of aldolase are titrated (*192*) or the perturbation of tyrosine absorption during the titration of ribonuclease histidines (*191*).

Donovan (*192*) discussed this problem in his study of the ionization of tyrosines and cysteines in aldolase. This enzyme becomes unfolded during the course of the titration; thus, a contribution to the difference spectrum is made by the change in the environment of the chromophores

202. M. J. Gorbunoff, *Biochemistry* **7**, 2547 (1968).
203. G. E. Perlmann, *JBC* **241**, 153 (1966).
204. M. Sokolovsky, J. F. Riordan, and B. L. Vallee, *Biochemistry* **5**, 3582 (1966).
205. G. E. Perlmann, *JMB* **6**, 452 (1961).
206. R. M. Herriot, *J. Gen. Physiol.* **45**, 57 (1962).
207. V. Frattali, R. F. Steiner, and H. Edelhoch, *JBC* **240**, 112 (1965).
208. M. J. Kronman, *in* "Fine Structure of Proteins and Nucleic Acids" (G. D. Fasman and S. N. Timasheff, eds.), p. 271. Marcel Dekker, New York, 1970.

when the pH is raised from neutrality. In this case, it was found that use of a reference solution at acid pH eliminated this complication since this enzyme is highly unfolded in that pH region as well and the unionized chromophores are in contact with solvent. A similar perturbing effect was found in the titration of the ribonuclease histidines (*191*). This system is a particularly good example of the manner in which progressively more detailed information may be obtained on the state of ionizing groups as different probes are applied.

Ribonuclease contains four histidines, two of which, His 12 and His 119, form part of the active site (*17*, *18*, *209–211*). Analysis of its acid binding titration curve showed that all four imidazoles ionize normally with an average intrinsic pK of 6.5 (*212*). Donovan (*191*) examined directly the histidine ionization, using difference ultraviolet spectroscopy which, in ribonuclease, gives a maximum at 237 nm for imidazole ionization. The data could be fitted either with a curve calculated for four groups ionizing with an intrinsic pK of 6.5 or with a curve for two sets of two groups each ionizing with apparent pK values of 6.2 and 6.8, as shown in Fig. 21a. The imidazole difference spectra, however, are weak and they could not be obtained directly. The pH dependence of difference spectra between 250 and 300 nm showed that the ionization of imidazole or α-amino groups perturbed the absorption properties of one or more of the abnormal tyrosines. Since tyrosine absorbs close to 235 nm, a tyrosine perturbation spectrum could be expected in that region as well, i.e., overlapping with the histidine ionization difference spectrum. Using the extent of perturbation above 250 nm, Donovan (*191*) calculated the expected tyrosine difference spectrum at 235 nm; the resulting negative band is shown in Fig. 22. Subtraction of this negative absorption from the experimental curve resulted in the sought imidazole difference spectrum shown by the dashed line of Fig. 22. Since imidazole ionization also perturbed some phenylalanine residues, Donovan (*191*) could conclude that these residues, as well as buried tyrosines, are located in the vicinity of one or more histidines.

Finally, the ribonuclease histidine ionization has been the subject of an NMR study by Meadows *et al.* (*195*, *196*). Using this method, it was possible to follow the ionization of the four groups individually since the chemical shifts of their C_2 and C_4 protons are different and each group gives an independent peak. The results are shown in Fig. 21b.

209. H. A. Scheraga and J. A. Rupley, *Advan. Enzymol.* **24**, 161 (1962).
210. R. Heindrickson, W. H. Stein, A. M. Crestfield, and S. Moore, *JBC* **240**, 2921 (1965).
211. R. E. Cathou and G. G. Hammes, *JACS* **87**, 4674 (1965).
212. C. Tanford and J. D. Hauenstein, *JACS* **78**, 5287 (1956).

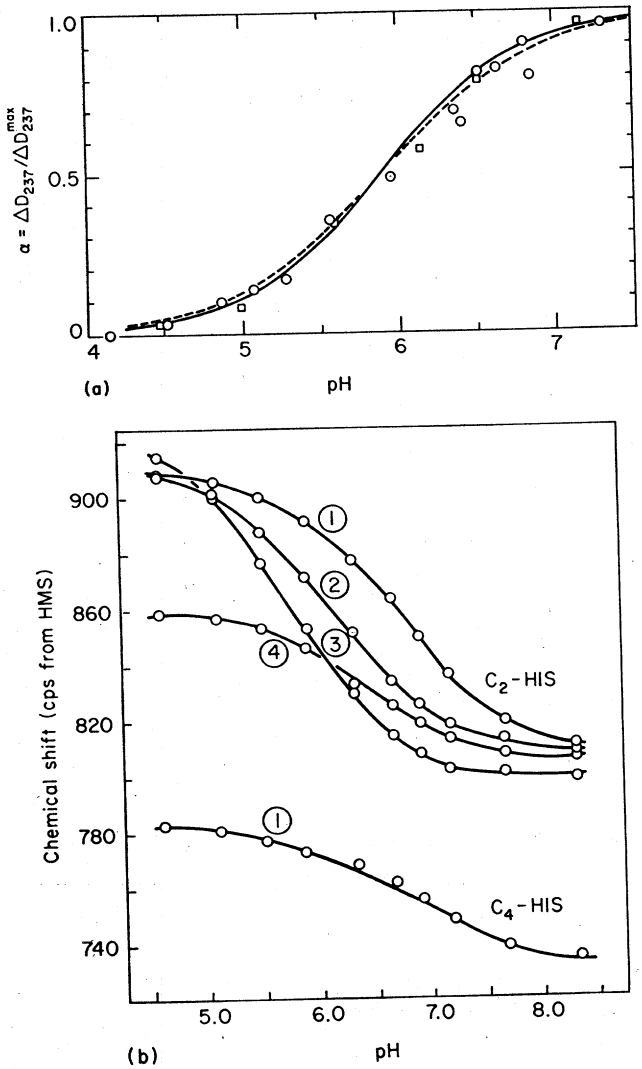


FIG. 21. Titration curves of the imidazole group of ribonuclease A. (a) Measured by ultraviolet difference spectra at 237 nm with a pH 4.1 reference uncorrected for superimposed perturbation of phenolic groups: (○) forward titration and (□) reverse titration from pH 6.5. The solid curve is calculated for four groups with an intrinsic pK of 6.5; the dashed curve is calculated for two groups with an intrinsic pK of 6.8 and two groups with an intrinsic pK of 6.2 [from Donovan (*191*)]. (b) Measured by NMR. The curves represent the variation of the chemical shift [expressed as cycles per second (cps) from a hexamethyldisiloxane (HMS) reference] as a function of pH for four C_2 hydrogen peaks and one C_4 hydrogen peak. The apparent pK's are for 32°C in 0.2 M deuteroacetate buffer [from Meadows *et al.* (*196*)].

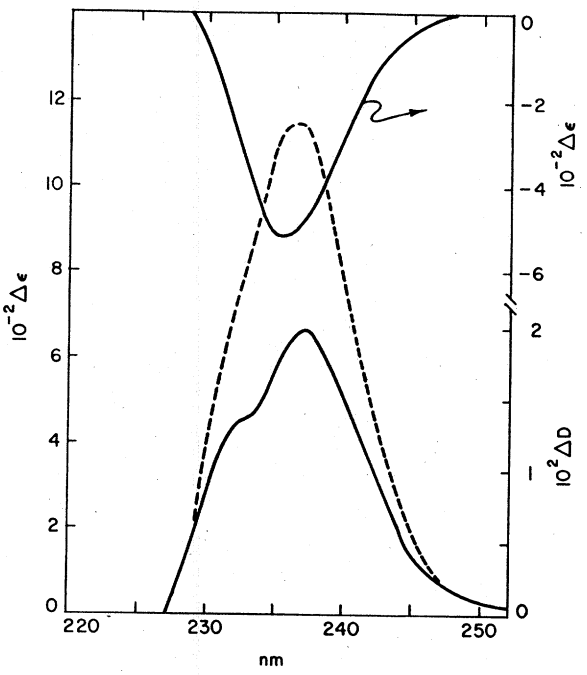


FIG. 22. Calculation of the corrected ultraviolet difference spectrum for the dissociation of protons from the four ribonuclease histidine residues. Lower solid curve: experimental difference spectrum for a pH 7.0 solution against a pH 4.1 reference; upper solid curve: calculated perturbation difference spectrum for affected tyrosines; dashed curve: correct imidazole difference spectrum [from Donovan (*191*)].

Four titration curves are obtained with pK values of (1) 6.7, (2) 6.2, (3) 5.8, and (4) 6.4. Carboxylmethylation of either His 12 or His 119 shifts peaks (2) and (3), indicating the mutual proximity in space of these two residues as can be expected from their presence within the active site; exchange of the His 12 C_2 proton for deuterium eliminated peak (2) permitting its assignment to residue 12; peak (4) was assigned to His 48 because of its anomalous chemical shift and line width, which is consistent with the buried state of this residue. This analysis established that the four histidines of ribonuclease have essentially normal pK values which, however, vary over nearly one pH unit. The pK values are 6.2 (His 12), 6.4 (His 48), 6.7 (His 105), and 5.8 (His 119). Similar histidine titrations have been performed with staphylococcal nuclease and lysozyme (*195*).

The histidine NMR spectra were further used by Roberts *et al.* (*213*)

213. G. C.-K. Roberts, J. Hannah, and O. Jardetzky, *Science* **165**, 504 (1969).

to probe the active site of ribonuclease with the spin-labeled inhibitor, 2,2,6,6-tetramethyl-4-hydroxypiperidin-1-oxyl monophosphate. Increasing amounts of the inhibitor resulted in the broadening of the C_2 peaks of His 12 and of His 119 (to a somewhat smaller extent), while His 105 was not affected, nor probably was His 48. Furthermore, the main aromatic region of the NMR spectrum remained unchanged. These results indicate that the unpaired electron of the inhibitor is relatively close to His 12, somewhat farther from His 119, and distant from the other two histidines as well as from the tyrosines and phenylalanines. Since addition of cytidine nucleotides to ribonuclease affects the NMR spectrum of phenylalanine 120 (*214*), it is evident that the free radical portion of the inhibitor does not lie on the cytidine binding site.

The abnormally titrating carboxyls of lysozyme have been the object of several studies. Donovan *et al.* (*172*) concluded from the titration curve that this enzyme contains at least one carboxyl with an abnormally high pK and one with an abnormally low pK. These groups have also been detected by the quenching of trytophan fluorescence (*162*, *163*). Carboxyl ionization may be examined directly by difference infrared spectroscopy (*193*). Protonated carboxyls give a difference band at 1710 cm^{-1}; ionized groups give a band at 1560 cm^{-1}. Difference infrared spectra, determined on lysozyme over a wide pH range, fully confirmed the conclusion on the presence of both types of abnormal carboxyls (*215*). Sulfhydryl ionization gives rise to a difference spectrum, maximal at 235 nm. Using this peak, Donovan (*192*) was able to establish that aldolase contains about six available and 21 buried cysteine residues.

Conformational transitions in enzymes are frequently reflected in a steepening of the dependence of their proton uptake on pH. Typical examples are the titration curves of ribonuclease (*173*, *212*) and carbonic anhydrase (*24*, *199*) in the alkaline region, where abnormal tyrosines become exposed to solvent, or the titration curve of carbonic anhydrase in the pH region between 5 and 4 (*199*) where seven previously buried histidines are liberated to ionization. Structural transitions of an enzyme may be characterized in detail by examining the pH dependence of the derivative with respect to pH of the proton binding h, i.e., of the buffering capacity of the protein, $\beta' = (\partial h/\partial \mathrm{pH})_{T,p}$ (*216*, *217*). At the point of conformational transition a maximum is obtained in this

214. D. H. Meadows and O. Jardetzky, *Proc. Natl. Acad. Sci. U. S.* **61**, 406 (1968).

215. J. A. Rupley and S. N. Timasheff, in preparation.

216. G. E. Perlmann, A. Oplatka, and A. Katchalsky, *JBC* **242**, 5163 (1967).

217. T. M. Birshtein and O. B. Ptitsyn, "Conformation of Macromolecules," Chapter 10. Wiley (Interscience), New York, 1966.

curve. Integration of the area under the curve gives Δh, i.e., the number of groups exposed during the conformational change. The temperature dependence of this displacement along the pH scale of the maximum position of β' results in the entropy and enthalpy of the transition. In this way, Perlmann *et al.* (*216*) probed the conformational transition of pepsinogen. Their results, shown in Fig. 23, indicate that a pH- and temperature-dependent transition is occurring. Integration under the individual curves showed that the three histidine residues of pepsinogen were being liberated to contact with solvent during the transition. The upward shift of the apparent pK of these groups was attributed to ion-pair formation between imidazolium and carboxylate ions in the zymogen.

The above examples have shown specifically how the structural environment of various types of ionizable groups may be probed by making use either of their spectra or of secondary effects which ionization of these groups generate in the enzyme, for example, the perturbation of tyrosine absorption by ionizing histidines in ribonuclease. Generally, probing is most successful when the state of a group is altered between "normal" and "abnormal" in the course of the process. In some specific cases, like the histidines of ribonuclease, the detected small differences in ionization

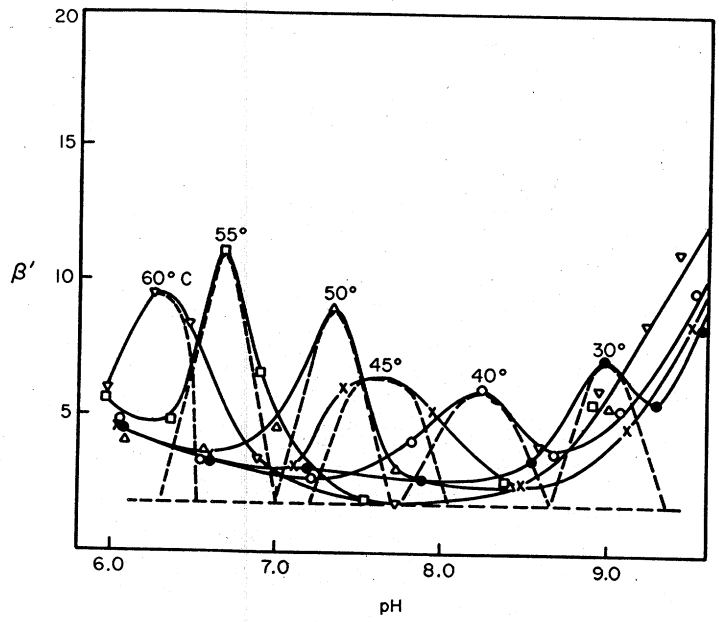


FIG. 23. Buffering capacity β' values derived from the titration curves of pepsinogen at various temperatures. The dashed lines under each curve indicate the pH limits used for the integration [from Perlmann (*216*)].

pK of individual "normal" groups reflect subtle differences in the environment of these groups and of their interactions with the environment. As the structures of enzymes become known in greater detail, it should become even more possible to analyze quantitatively these effects and, conversely, to use their occurrence as probes of detailed structural features.

IX. Geometry and Quarternary Structure

The overall geometry of enzyme molecules in solution may be probed with a variety of techniques, which measure either the hydrodynamic properties of these macromolecules or specific structural parameters. Hydrodynamic methods such as viscosity, flow birefringence, dielectric dispersion, and sedimentation velocity give little structural information beyond a very general description of the flexibility and degree of asymmetry of the molecules. For example, most globular proteins have intrinsic viscosities close to 3–4 cc/g, indicating that they are compact highly symmetrical molecules (*218*). Changes in this quantity are useful probes of conformational alterations without, however, giving any detailed structural information. For example, Conway and Koshland (*219*) have found that the process of binding a third molecule of DPN to tetrameric rabbit glyceraldehyde-3-phosphate dehydrogenase is accompanied by a change in intrinsic viscosity from 2.2 to 3.7 cc/g. Thus, while the structure remains globular, it becomes either more asymmetric or more flexible. Similarly, polarization of fluorescence has been used successfully to examine the general flexibility of some enzymes and the rigidity of quarternary structures formed of subunits (see Section VII); this technique, however, permits the drawing of conclusions concerning the motility or flexibility of specific regions of an enzyme by examining directly the rotational diffusion of the emitting chromophores within those regions.

The best method available at present for probing the geometry of macromolecules in solution is small-angle X-ray scattering. The development of absolute intensity equipment (*220–222*) has made it possible to

218. C. Tanford, "Physical Chemistry of Macromolecules," p. 394. Wiley, New York, 1961.
219. A. Conway and D. E. Koshland, Jr., *Biochemistry* **7**, 4011 (1968).
220. V. Luzzati, J. Witz, and R. Baro, *J. Phys. Phys. Appl.* **24**, 141A (1963).
221. O. Kratky, *Progr. Biophys. Mol. Biol.* **13**, 105 (1963).
222. H. Pessen, T. F. Kumosinski, S. N. Timasheff, R. R. Calhoun, Jr., and

determine directly a number of characteristic structural parameters (*223–227*). These are the molecular weight, the radius of gyration, the surface to volume ratio, the hydrated volume, and the degree of hydration. A combination of these parameters imposes strict limits on the geometry of the enzyme molecule. At higher angles, small-angle X-ray scattering curves display secondary maxima, which are highly sensitive to specific geometric details of the structure and enable further restriction of allowable structures of the macromolecules. Application of this technique to the absolute intensity scale has resulted in reasonable values for molecular parameters. For example, for ribonuclease a radius of gyration of 14.6 Å was measured (*222*); this should be compared with 15.0 Å calculated from the crystallographic data (*17, 18*). For lysozyme a molecular weight of 14,100 daltons was measured (*228*); the chemical value is 14,308 daltons.

Studies devoted to the probing of particular enzyme processes have been extremely limited up to now. Krigbaum and Godwin (*229*) examined the activation of chymotrypsinogen to chymotrypsin and concluded that this reaction does not result in gross structural changes. Their results gave almost identical radii of gyration and surface to volume ratios (S/V) for the zymogen ($R_g = 18.1$ Å, $S/V = 0.160$ Å^{-1}) and the product of slow activation, α-chymotrypsin ($R_g = 18.0$ Å, $S/V = 0.157$ Å^{-1}). The product of rapid activation, however, δ-chymotrypsin was found to be somewhat larger and more symmetrical than the precursor ($R_g = 19.0$ Å, $S/V = 0.146$ Å^{-1}).

The technique of small-angle X-ray scattering seems to be particularly well suited to the characterization of the assembly geometry of subunit proteins (*230, 231*). Such a study has been carried out by Sund *et al.* (*232*) on beef liver glutamate dehydrogenase as a function of

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enzyme concentration. It was found that this enzyme forms end-to-end aggregates which can be described best by the model of elliptical cylinders with long and short cross-section axes of 95 and 76 Å and a mass per unit length of 2340 daltons/Å. The cross section is independent of protein concentration, the length of the aggregates being linearly proportional to the molecular weight. The presence of a secondary maximum at an angle corresponding to a Bragg value of 56 Å indicates that the enzyme molecule is loosely built and possesses voids. With this geometric model it became possible to interpret quantitatively the hydrodynamic parameters of this enzyme (*233*). Furthermore, the knowledge from light scattering that the 2×10^6 daltons molecular species contains eight subunits (*234*) has permitted arrival at a geometry of the monomer which is best described as a globular structure with dimensions of 110, 95, and 76 Å along the three axes of the molecule.

X. Conclusions

In this chapter an attempt has been made to summarize by specific examples the type of structural information that may be obtained when enzymes are probed in solution by physical measurements and to stress the pitfalls that must be avoided. No attempt at an exhaustive coverage of available techniques was made. For example, the use of nuclear magnetic resonance as a conformational tool has been only mentioned (this technique, however, is discussed in some of its ramifications in Chapter 9 by Mildvan, this volume). Methods based on kinetic measurements have been totally excluded. Examination of the degree of success that has been achieved with various primarily spectroscopic techniques leads to the conclusion that in the present state of the art it is possible to gain a considerable amount of structural information by probing specific groups in various ways, as they are involved in interactions, either with other structural elements of the enzyme macromolecule or with ligands such as substrates, inhibitors, or chromophores artificially attached at selected positions. In general, interpretation of data obtained with any single technique is hazardous and extreme caution must be exercised in interpreting spectral and other results in terms of particular structures. The greatest amount of success has been achieved when several techniques were applied to the same question. Normally, the multitechnique approach gives as final result more information than the sum of the

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conclusions permitted by each technique individually. This approach allows and requires constant integration of all the pieces of information gleaned from individual experiments. It can be expected that the continuation of this approach with even more sensitive probes will bring its reward in terms of a progressively better and more detailed understanding of the manner in which enzymes and other biological macromolecules perform their various functions.